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SOME PROBLEMS IN THE EVALUATION OF DIFFERENTIAL GROWTH IN THE RAT'S SKULL

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Introduction

Moss and Baer (1956) have recently investigated the growth of the rat's skull by a method of differential growth analysis. In the course of an investigation into the influence of the thyroid gland on growth of the rat's skull, measurements were made on the skulls of a series of maturing rats, and these data are used here for a critical examination of the method of differential growth analysis by means of a double logarithmic plot as used by Moss and Baer and other workers (Moss, 1954; Moss, Noback & Robertson, 1956). These authors plotted their measurements of one bodily dimension (ordinate) against another (abscissa). By this means they sought to describe the relative growth rates of the two dimensions, and often defined two phases of growth, separated by a so-called "interphase" (a critical point in time at which there is an abrupt change in growth rate). It is the purpose of the present communication to examine critically the appropriateness of this representation of growth along the two axes. In particular we shall examine the justification for Moss and Baer's procedure of fitting (empirically and not algebraically) a separate straight line for each of the two putative phases of growth.

Material and Methods

Forty albino rats, of the Wistar strain kept in this laboratory, were used. Groups of five were killed at birth, at ten, fifteen, twenty, thirty, forty, sixty and eighty days of age. Each rat in a group of five came from a different litter; the sexes were mixed indiscriminately. Immediately after death radiographs were taken of each rat, using a tube-plate distance of three feet, with the rat in three positions:—

1. Lying ventrally on the plate with a cotton thread passing over

and pressing on the back of the neck, to keep the cranial base parallel to the plate.

2. Lying on its side with the two external auditory meatuses on the same vertical line.
3. Lying ventrally on the plate with the thorax raised on a piece of cotton wool so that the nostrils were on the plate, and in the same vertical plane as the external auditory meatuses (thus giving a postero-anterior view of the skull).

Measurements were made on the radiographs using calipers with a vernier scale measuring to 0.1 mm. With a tube-plate distance of three feet errors due to magnification or distortion of the image amounted to about 1%, which was of the same order of magnitude as possible errors of measurement and were therefore ignored.

Method of Differential Growth Analysis Employed

The method of growth analysis which we used was to plot measurements of skull dimensions on a logarithmic scale against age in days on an arithmetic scale. When this is done the slope of the curve at any age is proportional to the specific growth-rate of the dimension at that age; while changes in the slope of the curve represent changes in the specific growth-rate of the dimension. The specific growth-rate of the dimensions at any age is therefore directly proportional to the tangent of the angle which the curve makes with the abscissa at that age. When the growth of two dimensions is plotted against age on the same semi-logarithmic paper the ratio k (the 'growth constant' or 'growth ratio') of their specific growth-rates at any age is given by the ratio of the tangents of the two curves at that age.

If the same two dimensions are plotted one against the other on a double logarithmic scale the same ratio (k) is then given by the tangent of the curve thus obtained. This is the method first extensively employed by Huxley (1932) and used by Moss and Baer (1956) on the rat's skull.

The specific growth rate (the mean increment of the natural logarithm of the dimension per unit time) of a dimension, between time t_1 and t_2 is indicated by the slope of the line joining the two points when they are plotted semi-logarithmically. If the interval between t_2 and t_1 is large, the mean specific growth rate so defined gives only an average estimate of growth during this period. In fact we here

assume that the specific growth rate is uniform over the period in question. This assumption will be valid only if $(t_2 - t_1)$ is small; the more widely spaced, the less justification we have for making this assumption. We need therefore, a means of estimating the change in specific growth rate at intervals during the period from t_1 to t_2, i.e. the specific growth rate over a smaller period of time. To derive the specific growth rate between time t and $t + \triangle t$, where $\triangle t$ tends to zero, an alternative procedure must be used. A useful technique which has been recommended to us, is based upon the least squares method of fitting segments of polynomials to a limited number of consecutive points (usually odd) and calculating the first derivative of the fitted polynomial at a given point (usually the mid-point). Suitable formulae for determining this derivative directly can be found in text books on numerical calculus (e.g. Milne, 1949): by recourse to this method we can estimate the appropriate specific growth rates algebraically. Although 5, 7 or more points may be used it was only considered justified to use the following 3-point parabolic formulae in this study, owing to the simple nature of the data. The natural logarithm (y) of the dimension to be studied is found for each age and the 3 point derivative formulae then used are as follows $(h = 10$ days).

1. For the slope of the segment from t_0 to $t_0 + \triangle t_0$,

$$\frac{dy}{dt_0} = \frac{1}{2h} \quad (-3y_0 + 4y_1 - 1y_2)$$

2. For the segment from t_1 to $t_1 + \triangle t_1$,

$$\frac{dy}{dt_1} = \frac{1}{2h} \quad (-1y_0 + 1y_2)$$

3. For the segment from t_2 to $t_2 + \triangle t_2$,

$$\frac{dy}{dt_2} = \frac{1}{2h} \quad (+1y_0 - 4y_1 + 3y_2)$$

Formulae 1 and 3 were used to estimate the specific growth rates of segments about the first and last points in a series, and formula 2 for the segments at all other points. In order to use these formulae the measurements must be evenly spaced in time. We have, therefore, estimated the values of 50 and 70 days by averaging the figures for 40 and 60 and 60 and 80 days respectively.

This technique has been used to estimate the relative growth rates and hence the values of k, in Table 2.

Results

Figure 1 shows the dimensions which were measured. The mean values of all these measurements, with their standard deviations at each age, are given in Table 1.

1. *Differential growth between cranial and facial portions of the skull base.*

Figure 2 shows the relation between the specific growth-rates of the dimensions basion-prosphenion and prosphenion-nasion lengths from birth to 80 days. From birth to between 30 and 40 days basion-prosphenion length is slightly greater than prosphenion-nasion length, though the latter has the higher specific growth-rate, and comes to equal the former length between 30 and 40 days; by 80 days it is 1.24 mm. greater ($P < 0.001$). These results have been related to the different rates of development of the neural and facial skeletons (Moss and Baer '56).

2. *Differential growth of the bones of the cranial base*

Up to 20 days the basi-sphenoid length increases more rapidly than basi-occipital length (Fig. 3b) and hence its specific growth rate exceeds that of the basi-occipital length. The ratio k, of the specific growth rates is therefore greater than 1 (Table 2). After this time the two curves ran almost parallel (k varies between 1.3 and 0.7) with the suggestion that the rate of increase of the basi-sphenoid length is gradually falling behind that of the basi-occipital length. The findings of Moss and Baer (1956) that the basi-sphenoid and pre-sphenoid lengths have similar specific growth-rates is implied by our measurements (Table 1).

The basi-occipital bone grows relatively faster in width than in length during the first 10 days. Thereafter its length and width have the same specific growth rates since there are no significant differences between the increment of the logarithm of each dimension. The finding of Moss and Baer (1956) that the specific growth rate of maximum basi-occipital width is consistently greater than that of maximum basi-sphenoid width is confirmed. The basi-sphenoid bone grows more rapidly in length than in width up to 30 days (Table 1);

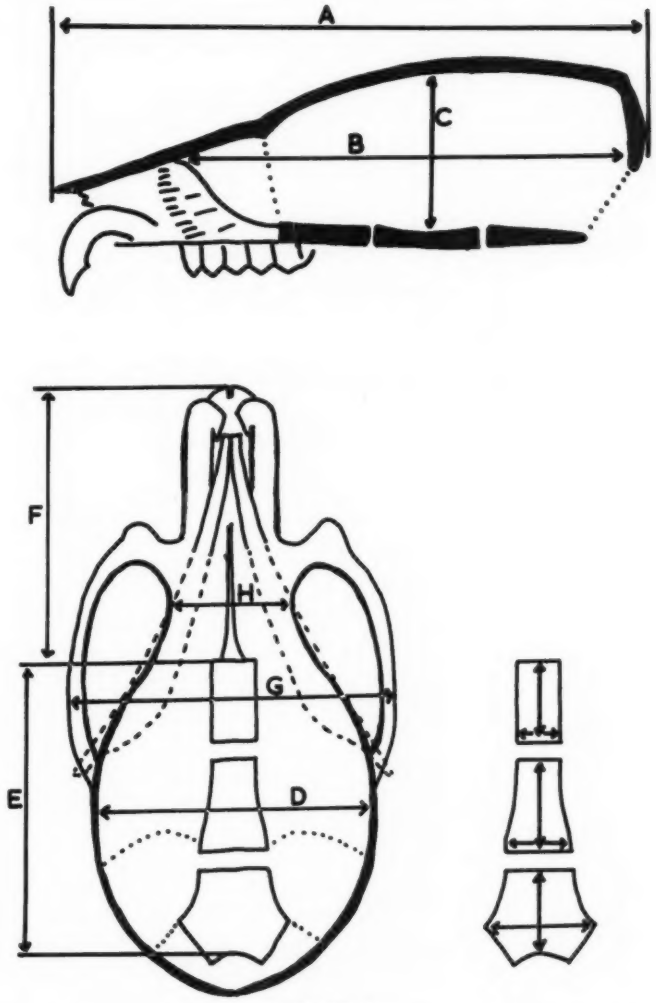


FIGURE 1

Diagrams of the rat skull seen from laterally (above) and from above (below left), and of basi-occipital, basi-sphenoid and pre-sphenoid bones (below right), indicating the measurements which were made.

A, total skull length; B, endocranial length; C, endocranial height; D, endocranial width; E, basion-prosphenion; F, prosphenion-nasion; G, bizygomatic width; H, least frontal width.

TABLE 1

MEAN SKULL DIMENSIONS ± STANDARD DEVIATIONS (ALL MEASUREMENTS IN MILLIMETRES)

Age (days)	0		10		15		20		30		40		60		80	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Total skull length	14.78	0.35	24.20	1.41	28.06	1.47	30.54	0.80	34.76	0.62	37.02	0.44	40.88	1.02	42.74	1.52
Endocranial length	11.22	0.28	17.04	0.92	19.36	0.92	20.50	0.33	21.64	0.41	22.50	0.34	23.82	0.58	24.16	0.86
Endocranial width	8.12	0.32	12.38	0.47	13.71	0.33	13.91	0.39	14.34	0.24	14.85	0.26	15.05	0.33	15.16	0.19
Endocranial height	5.16	0.16	7.88	0.32	8.92	0.20	8.92	0.27	9.22	0.21	9.50	0.19	9.62	0.32	9.94	0.48
Basion-prosphenion	7.42	0.17	11.12	0.60	12.68	0.62	14.14	0.31	15.60	0.29	16.68	0.13	18.42	0.79	19.62	0.81
Prosphenion-nasion	6.22	0.29	10.25	0.66	12.06	0.72	13.14	0.52	15.48	0.35	17.24	0.48	19.56	0.34	20.86	0.80
Basi-occipital length	2.40	0.24	3.38	0.12	3.90	0.23	4.42	0.12	4.98	0.20	5.32	0.12	6.00	0.30	6.32	0.22
Basi-occipital width	2.46	0.27	4.24	0.31	4.78	0.32	5.32	0.12	6.18	0.38	6.34	0.21	7.66	0.74	8.20	0.22
Basi-sphenoid length	1.50	0.12	3.22	0.19	4.04	0.40	4.76	0.27	5.38	0.13	5.88	0.07	6.64	0.21	6.94	0.37
Basi-spenoid width	1.80	0.37	2.66	0.18	2.96	0.15	3.30	0.15	3.38	0.15	3.64	0.12	4.08	0.19	4.20	0.26
Presphenoid length	1.44	0.36	3.08	0.25	3.92	0.31	4.42	0.15	4.94	0.27	5.26	0.08	5.42	0.44	6.14	0.34
Presphenoid width	1.56	0.27	1.88	0.17	2.30	0.21	2.14	0.22	2.30	0.23	2.34	0.14	2.72	0.04	2.70	0.02
Cribriform plate width	3.72	0.10	4.92	0.17	5.10	0.17	5.16	0.08	5.18	0.12	5.14	0.05	5.12	0.20	5.02	0.12
Bizygomatic width	9.36	0.41	14.02	0.59	15.36	0.61	16.10	0.36	18.18	0.32	19.48	0.20	21.26	0.77	22.86	0.87
Least frontal width	4.32	0.07	6.54	0.08	7.08	0.21	7.30	0.11	7.74	0.17	8.24	0.14	9.12	0.25	9.54	0.25

TABLE 2

SPECIFIC GROWTH RATES OF BASI-SPHENOID AND BASI-OCCIPITAL LENGTHS CALCULATED FROM A 3 POINT DERIVATIVE FORMULA

Age (days)	Dimension (mm)		Log_e Dimension (y)		Specific growth rate dy/dt mm/mm/day		k
	Basi-sphenoid	Basi-occipital	Basi-sphenoid	Basi-occipital	Basi-sphenoid	Basi-occipital	
0	1.50	2.40	0.405	0.876	0.095	0.038	2.5
10	3.22	3.38	1.169	1.218	0.058	0.031	1.9
20	4.76	4.42	1.560	1.486	0.026	0.019	1.4
30	5.38	4.98	1.683	1.605	0.011	0.009	1.2
40	5.88	5.32	1.772	1.672	0.008	0.006	1.3
50	6.26*	5.66*	1.834	1.733	0.007	0.006	1.2
60	6.64	6.00	1.893	1.792	0.004	0.004	1.0
70	6.79*	6.16*	1.916	1.818	0.002	0.003	0.7
80	6.94	6.32	1.937	1.844	0.002	0.003	0.7

* Estimated values.

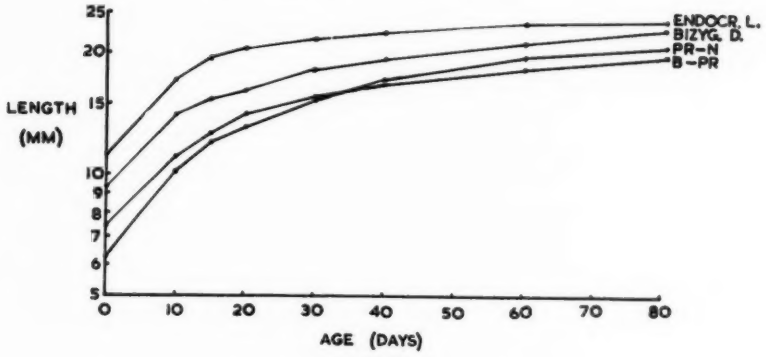


FIGURE 2

Certain skull dimensions, on a logarithmic scale, plotted against age on an arithmetic scale.

Endocr. L., endocranial length; Bizyg. D., bizygomatic width; Pr-N, prosphenion-nasion; B-Pr, basion prosphenion.

thereafter the logarithms of the increments in length and width are not markedly different so that the specific growth rates are approximately equal. This finding also applies to the pre-sphenoid bone.

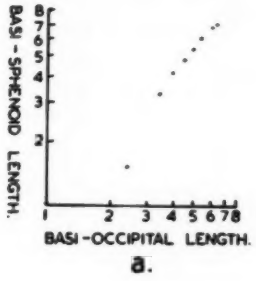
The relative percentages of basion-prosphenion length occupied by the basi-occipital, basi-sphenoid and pre-sphenoid bones are shown in Table 3. From this table it can be seen that the basi-occipital

TABLE 3

STRUCTURES COMPRISING THE CRANIAL BASE, EXPRESSED AS A PERCENTAGE OF THE OVERALL LENGTH OF THE BASE AT DIFFERENT AGES

Age (days)	Basi-occipital %	Basi-sphenoid %	Pre-sphenoid %	Cartilage
0	32.3	20.2	19.4	28.1
10	30.4	29.0	28.1	12.5
20	31.3	33.7	31.3	3.7
30	31.9	34.5	31.7	1.9
80	32.2	35.4	31.3	1.1

bone already occupies its definitive proportion of basion-prosphenion length at birth, while the basi-sphenoid and pre-sphenoid bones do not begin to attain their definitive proportions until about 20 days. During the period 0–20 days the proportion of cartilage in this part of the cranial base is steadily decreasing.



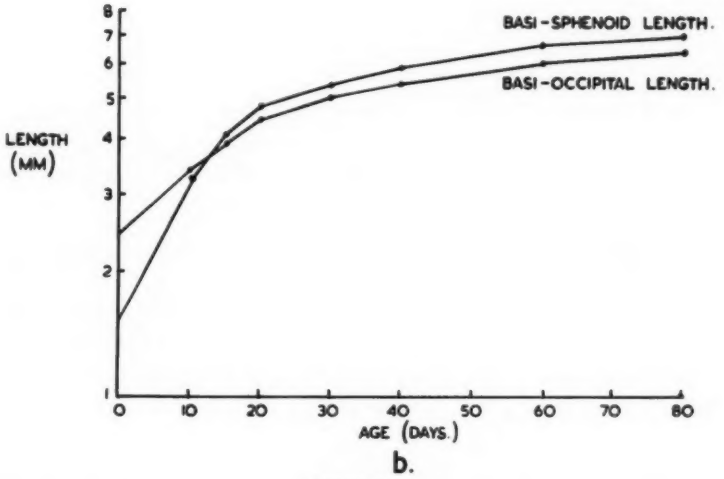


FIGURE 3

(a) Basi-occipital length plotted against basi-sphenoid length on a double logarithmic grid;
(b) The same two dimensions plotted on a logarithmic scale against time on an arithmetic scale.

3. *Differential growth of the cranial cavity*

Figure 4 is a semi-logarithmic plot of endocranial length, width and height against time. The specific growth-rates of these 3 dimensions are similar up to 15 days; thereafter, while endocranial height and width continue to have the same specific growth rates that of

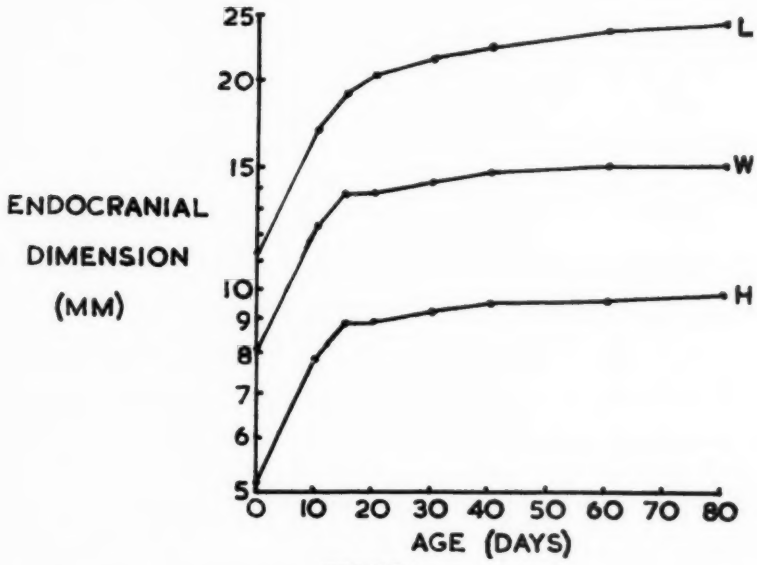


FIGURE 4
Endocranial length (L), width (W) and height (H) on a logarithmic scale plotted against age on an arithmetic scale.

the endocranial length is consistently higher up to 80 days. As a consequence there is a gradual increase of endocranial length relative to width and height with a corresponding reduction in the endocranial index, most marked between 15 and 20 days (Table 4).

TABLE 4

ENDOCRANIAL INDEX $\left(\frac{\text{Endocranial width} \times 100}{\text{Endocranial length}}\right)$ AT VARIOUS AGES

Age (days)	0	10	15	20	30	40	60	80
Endocranial Index	72.4	72.7	70.8	66.9	66.3	66.0	63.2	62.7

If the increase of endocranial length with time is compared graphically (Fig. 2) to that of basion-prosphenion length, also an endocranial dimension, it can be seen that whilst up to 15 days the curves are parallel, indicating that the specific growth-rates are identical, between 15 and 80 days the curve for basion-prosphenion length ascends

more steeply than that for endocranial length, indicating that its specific growth-rate is consistently higher. This is associated with a movement backwards of the basion relative to the endocranial cavity as a whole, with the consequence that the foramen magnum comes to face more backwards as the skull increases in size.

4. *Growth of other dimensions measured*

The width of the cribriform plate was measured on the postero-anterior radiographs of the skulls, on which it was clearly visible. This dimension ceased to enlarge significantly after 15 days (Table 1) and thus ceased growth earlier than any other cranial dimension measured.

Two other width measurements were studied, bizygomatic width and least frontal width. It can be seen (Fig. 2) that the slope of the curve of bizygomatic width and hence the specific growth rate is very similar to that of the cranial portion of the base (basion-prosphenion), rather than to that of the facial portion (prosphenion-nasion), although the opposite might have been expected, since bizygomatic width is presumably related to the size of the muscles of mastication, and hence to the size of the jaws.

The rate of increase of the least frontal width is similar to that of the bizygomatic width up to 10 days (Table 1) so that the specific growth rates are equal. After 10 days the specific growth rate of least frontal width declines sharply in comparison with that of bizygomatic width, presumably in relation to the cessation of growth in width of the cribriform plate.

Discussion

The significance of an "interphase" on a double logarithmic plot may conveniently be discussed in relation to the growth in length of the basi-occipital and basi-sphenoid bones. Moss and Baer (1956) find a clear "interphase" in the differential growth curve of these two bones occurring when the basi-occipital bone is about 5 mm. long. They cite this as evidence of a "critical developmental stage." They calculate the ratio, k, of the two specific growth rates to be 1.5 before the interphase and 0.67 after the interphase when ". . . the ratio favours the growth of the basi-occipital length."

We have plotted our data for the two dimensions on a similar double logarithmic grid (Fig. 3a) and also as individual curves on a semi-logarithmic grid in respect of age (Fig. 3b).

It can be seen from Fig. 3c that after approximately the 30th day of age the curves for the two dimensions appear to be parallel. This appearance is probably deceptive since the calculated values (Table 2) for k, from birth to 80 days are continuously variable. There therefore seems no justification for giving a single value to k for the period 0–20 days (*which is the effect of fitting a straight line to these points on a log-log graph*) or indeed for any other period. It would seem that a continuous curve is more appropriate for the log-log data, suggesting that a critical point of inflexion is a methodological artifact. Reeve and Huxley (1945, p. 144) state that "Apart from Crustacea, critical points have occasionally been described in the allometric growth of various vertebrates but their validity as against smooth curves is sometimes in doubt."

Before describing critical "interphases" or points of inflexion on allometric plots it is essential to demonstrate that the data are best fitted by two or more intersecting straight lines rather than a smooth curve. Richards and Kavanagh (1945) have described ways of doing this. We would suggest that of these methods, that of plotting the data for both dimensions individually on a single logarithmic plot is the simplest and most illuminating graphic method. It has the additional advantage that it gives information as to the actual changes of specific growth rate in respect of time for each dimension. To quote Richards and Kavanagh (1945, p. 199), "It (k) is not always a constant, yet may remain so while the specific growth rates are themselves changing in such a manner as not to affect their ratio. In the latter case a plot of the specific growth rates with respect to time is more informative" (than an allometric plot).

In the example we have chosen, such a plot indicates that the basi-sphenoid bone is growing relatively much more rapidly than the basi-occipital bone at birth, but that its specific growth-rate continuously slows until, after about 20 days, the two bones have the same or perhaps slowly inverting specific growth rates. The morphological explanation of this is given above (Table 3) and is that the basi-occipital bone already occupies its definitive proportion of basion-prosphenion length at birth while the basi-sphenoid bone does not attain its definitive proportion of basion-prosphenion length until about 20 days. This finding can itself be explained embryologically; the parachordal (basi-occipital) part of the cranial base develops before the prechordal part and is therefore more advanced in develop-

ment at any given age. This is in accordance with the law of developmental direction (Kingsbury, 1924; Ford, 1956).

It may be noted in conclusion that the rat's skull shows several features in its growth which are found in the human skull, and may be general mammalian characteristics. Two of these features are:—

(1) The early cessation of growth of the cribriform plate, while the rest of the neurocranium is still growing; growth of the plate stops at about 15 days in the rat and at under 2 years in man (Ford, 1958), and this suggests the possibility that the olfactory bulbs may complete their growth well before the rest of the brain.

(2) The shift backwards of the basion in relation to the rest of the neuro-cranium during post-natal growth.

Summary

1. The growth of the rat's skull between birth and 80 days has been investigated by means of measurements made on radiographs.

2. Certain methods of differential growth analysis are critically reviewed, and it is concluded that whilst comparison of the growth-rates of two dimensions by plotting them against each other on a double logarithmic grid is valuable for showing up sharply changes in differential growth, it is not a good method of estimating the ratio (k) of the two specific growth rates. This (k) is better indicated by plotting both dimensions against time on a single logarithmic grid, and calculated numerically by the use of methods indicated in the text.

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References

1. Ford, E. H. R. 1956. The growth of the foetal skull. *J. Anat.*, **90**, 63-72.
2. ———. 1958. Growth of the human cranial base. *Amer. J. Orthodontics*, **44**, 498-506.
3. Kingsbury, B. F. 1924. The significance of the so-called law of cephalocaudal differentiation. *Anat. Rec.*, **27**, 305-321.
4. Milne, W. E. (1949). Numerical calculus, p. 97: Princeton University Press, Princeton, New Jersey.
5. Moss, M. L. 1954. Differential growth analysis of bone morphology. *Am. J. Phys. Anthrop.* **12**, 71-76.

6. ————, & Baer, M. J. 1956. Differential Growth of the Rat Skull. *Growth*, **20**, 107-120.

7. ————, Noback, C. R., & Robertson, G. G. 1956. Growth of certain human fetal cranial bones. *Amer. J. Anat.*, **98**, 191-204.

8. Reeve, E. C. R., & Huxley, J. S. 1945. Some patterns in the study of allometric growth; pp. 121-156 in 'Essays on Growth and Form presented to D'Arcy Wentworth Thompson.' Clarendon Press, Oxford.

9. Richards, O. W., & Kavanagh, A. J. 1945. The analysis of growing form; pp. 188-230 in 'Essays on Growth and Form presented to D'Arcy Wentworth Thompson.' Clarendon Press, Oxford.

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VASCULOGENESIS IN THE ADENOHYPOPHYSIS OF THE DEVELOPING CHICK EMBRYO[1]

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INTRODUCTION

THE pituitary is an exceedingly important constituent of the adult endocrine system. It is the site of production of trophic hormones which affect the synthetic abilities of other endocrine glands. Such trophic secretions are in turn dependent upon the secretory products of the other endocrines. The development of pituitary-target gland axes has been studied in detail in the developing chick embryo (Willier, 1955).

The time of initial function of the pituitary gland of chick embryos has been studied by means of cytological, histochemical and physiological techniques. The results vary according to the methods employed by various investigators (Tixier-Vidal, 1954).

Rahn (1939), using a modified Severinghaus technique, demonstrated that acidophils and basophils first appear in the anterior pituitary of 8-day chick embryos. Payne (1946), however, did not identify acidophils in embryos younger than 10 days. By means of the Bodian silver technique, Dawson (1947) noted argyrophilic cells in the anterior lobe of the embryonic chick pituitary on day 8. Wingstrand (1951) demonstrated silver positive cells in the anterior pituitary of chick embryos on the 6th day of incubation. These silver reducing cytoplasmic granules were considered by the author to have functional significance. However, he found that the first distinct basophils and acidophils did not appear until day 11. Similarly P.A.S. positive cells appear first on day 6 of incubation, although there is a decided increase in numbers of these elements on day 11 (Aronsson, 1952).

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Physiological data are also in disagreement. Pituitary ablation experiments (Fugo, 1940) as well as chorio-allantoic transplant studies (Martindale, 1941) have indicated that the anterior hypophysis of chick embryos is functional on or around day 11. Case (1952) observed that there was an arrest in weight increment of the adrenals, and retardation of rate of accumulation of adrenal ascorbic acid in hypophysectomized chick embryos beginning on day 13. Similar decapitation experiments by Konigsberg (1954) resulted in a pronounced hyperglycemia beginning on the 8th day of incubation. Studitskii (1946) concluded from experiments involving 8-day pituitaries that the gland is functional at this time. Chen, Oldham and Geiling (1940) injected saline suspensions of 5-day chick pituitaries into hypophysectomized frogs and, on the basis of melanophore-expanding hormone action, concluded that pituitary function in the chick begins in 5-day embryos. Rahn (1941) made the same observation using the *Anolis* lizard test.

In endocrine glands, other than the pituitary, there is evidence of a correlation between intrinsic vascular pattern and initiation of functional activity. Flint (1900) demonstrated in the adrenal of the fetal pig and dog, a change from a closely woven, irregular plexus of capillaries to a longitudinally oriented pattern of blood vessels. Similar changes in vascular patterns in adrenals of neonatal rats and mice were noted. These changes suggested a correlation between time of functional onset and an orientation of blood vessels which is more conducive to efficient blood flow (Gersh and Grollman, 1941). In the albino rat (Hard, 1944) the beta cells of the pancreas are first identified in 15½ day fetuses within a few hours after a capillary network has developed in the larger islets. Beta granules soon accumulate on the side of the cell toward the capillary network.

More recently, Thommes (1958) found that hypophysectomy prevents the normal progressive change from a distinctly sinusoidal pattern to a capillary pattern in the thyroid of the chick embryo.

These correlations between vascular change and functional differentiation suggest that similar correlations might be found in the developing pituitary gland. It is the purpose of the present investigation to describe vasculogenesis in the pituitary of the normal chick embryo and to compare these changes in vascularity with the known changes in histogenesis and functional activity of this gland.

Materials and Methods

All embryos were obtained from White Leghorn eggs incubated at 38.3 ± 1.1 degrees C. in forced draft incubators. The ages of all specimens represent the actual time the eggs remained in the incubator.

Injections of undiluted India ink were made by means of fine capillary pipettes. After candling the eggs to locate the major blood vessels in the extraembryonic membranes, the shell was carefully removed, the capillary tube gently inserted into a blood vessel, and the India ink introduced by gentle pressure. The capillary tube was then withdrawn and the eggs left undisturbed for a few minutes to permit the ink to circulate uniformly to all parts of the body.

Injected embryos were immediately freed from all membranes and surrounding extraembryonic material, washed in saline and fixed in Bouin's solution. Preserved specimens of embryos were prepared at daily intervals from the 3rd through the 18th day of incubation. Eighty embryos were successfully injected.

The younger embryos, from 3 to 8 days of incubation, were stained *in toto* with borax carmine. The heads of 9-day specimens and older were removed and trimmed to facilitate penetration of the stain. Subsequently, tissues were dehydrated, cleared, and embedded in nitro-cellulose. The tissues were section at 60μ and mounted serially according to the method of Feeney (1944). All photomicrographs were made with Kodak Plus X film.

Observations

The pituitary gland of the chick embryo has a double origin. The anterior lobe arises as a median tubular invagination (Rathke's pouch) of the ectoderm of the ventral surface of the head immediately in front of the oral plate (20 somite stage: 48–53 hours). The posterior lobe arises from an evagination of the floor of the diencephalon, the infundibulum. By growth inward, Rathke's pouch contacts the infundibulum at the 30 somite stage (52–64 hours). At about the 36 somite stage (72 hours) the distal portion of Rathke's pouch flattens and gives indication of branching. Subsequently it becomes much branched and quite massive, uniting with the infundibulum to form the hypophysis (Hamilton, 1952).

Vascularization of the anterior, glandular portion of the pituitary

gland or adenohypophysis of the chick embryo begins on day 6 of incubation. At this time a small number of capillaries, arising in the ventral wall of the diencephalon, penetrate the proliferating cellular cords of the gland. These vessels arch over and around the cords, uniting at the ventral posterior aspect of the gland in close association with the forming venous sinus. Vessels within the gland are sinusoidal, small in size, and randomly arranged (Fig. 1).

By the following day vascularity has increased, keeping pace with the rapidly proliferating gland. The greater number of sinusoids present at this time form a scant and irregular network between the cellular cords. The calibre of these blood vessels remains the same and a few are seen emptying into the ventral venous sinus (sinus cavernosus) (Fig. 2). It is at this stage of development that the glandular mass is divided into cephalic and caudal portions by the growth of the two internal carotid arteries and their connective.

By day 8 the pituitary gland has increased in size considerably with a proportionate increase in numbers of anastomosing sinusoids (Fig. 3). The vascular network does not show any intrinsic change, although many of the capillaries have become associated with the sinus cavernosus which has expanded and now circumscribes the entire gland.

On the 9th day of incubation the pituitary gland separates from the ventral aspect of the diencephalon with which it has been closely associated prior to this time (Fig. 4). This separation exerts a "pull" on the supplying blood vessels forming the portal system which has been described by Wingstrand (1951) and others. The area occupied by this system of vessels has been termed the *eminentia meridiana* (Wingstrand, 1951). The portal vesels from the eminentia are larger than the sinusoids within the gland. They enter the central mass of the cephalic lobe, arching over and around the cellular cords.

In pituitaries of 10-day embryos there is an increase in diameter not only of the centrally located vessels, but also in some of the more peripherally placed sinusoids (Fig. 5).

By day 11, an increase in size and number of sinusoids is apparent in all regions of the gland (Fig. 6). The resulting rich vascular plexus is in striking contrast to the sparse vascular network present on day 10.

This vascular transition is effectively completed in pituitaries of

12-day chick embryos (Fig. 7). Vascularization reaches a level of development which is independent of the size of the gland. Sections of 12-day pituitaries show that the total amount of vascular tissue closely approximates the total amount of glandular tissue. As a result, the glandular cords are in intimate contact with the sinusoidal vessels. This is essentially the definitive pattern.

From the 13th through the 18th days of incubation (Figs. 8, 9, 10), the volume relationship between vascular and glandular elements changes in favor of the glandular tissue. However, the established vascular pattern is not appreciably altered.

Discussion

Functional interrelationships between the anterior pituitary and other endocrine glands (thyroid, adrenals, gonads) have been shown to occur in the developing chick embryo (Willier, 1955).

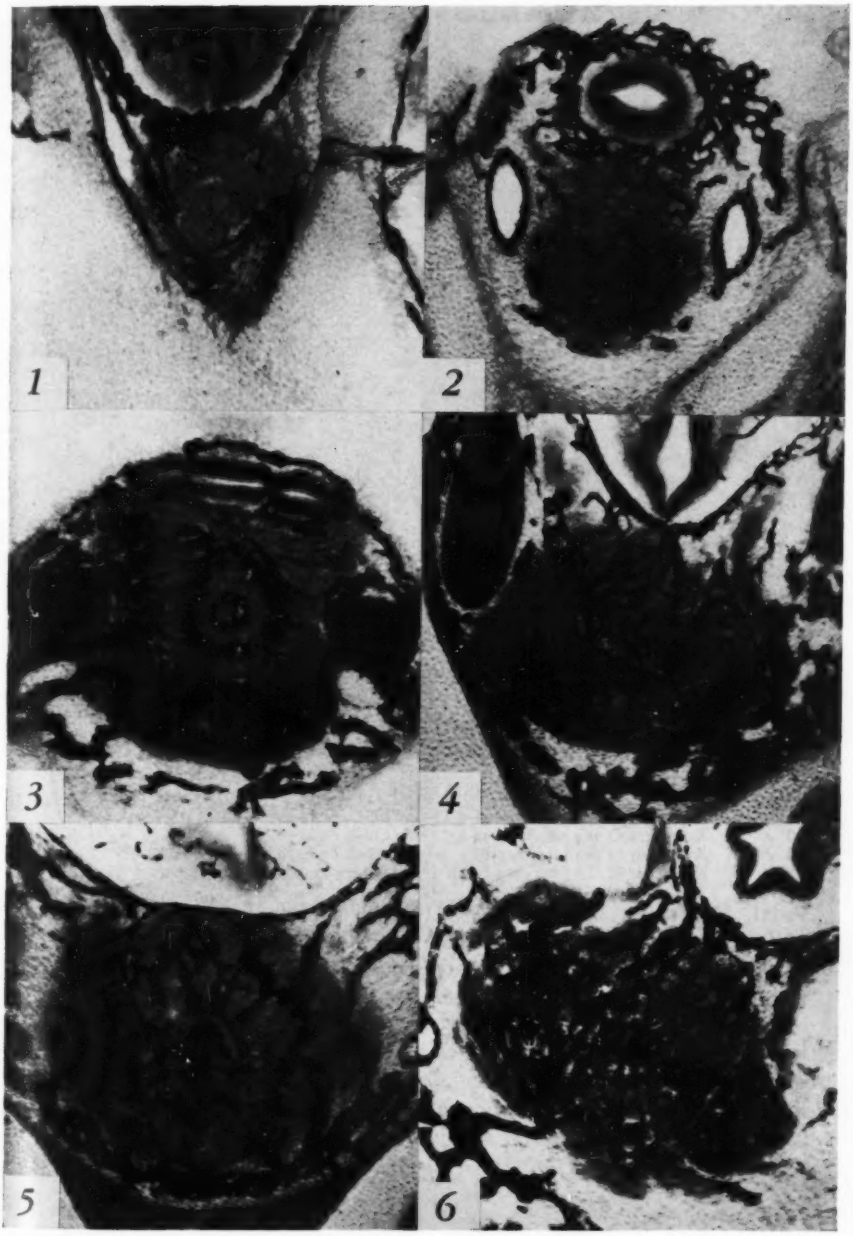
Physiological and cytological data regarding the time of onset of function of the adenohypophysis, as well as information pertaining to vasculogenesis of this gland has been summarized in Table 1. Present observations are included in the table and are distinguished by capital letters.

Observations made during the course of this study demonstrate the presence of a transitory phase in the vasculogenesis of the developing chick pituitary. This change begins on the 10th day and is completed by the 12th day of incubation. The transition from a somewhat scant plexus of sinusoids to an elaborate vascular network is indicative of a decided increase in the amount of blood passing through the gland. Such a change is undoubtedly favorable to an increase in the amount of raw material brought to the gland as well as to a more efficient distribution of trophic hormones produced by the pituitary.

Present observations can be correlated with the results of the studies of Fugo (1940) and Martindale (1941) which have indicated that T.S.H. is initially present on the 11th day of incubation. The above vascular change corresponds in time with histological changes described by many authors. Wingstrand (1951) observed the first distinct basophils and acidophils in the cephalic lobe of the adenohypophysis of *Gallus gallus* on the 11th day of incubation. Similarly, cells which respond positively to P.A.S., P.A.F. and azan stains have been reported to appear for the first time on this same day (Tixier-

PLATE I.

Fig. 1. X-section of the pituitary of a 6-day chick embryo. 96 ×
Fig. 2. X-section of the pituitary of a 7-day chick embryo. 96 ×
Fig. 3. X-section of the pituitary of an 8-day chick embryo. 96 ×
Fig. 4. X-section of the pituitary of a 9-day chick embryo. 96 ×
Fig. 5. X-section of the pituitary of a 10-day chick embryo. 96 ×
Fig. 6. X-section of the pituitary of an 11-day chick embryo. 96 ×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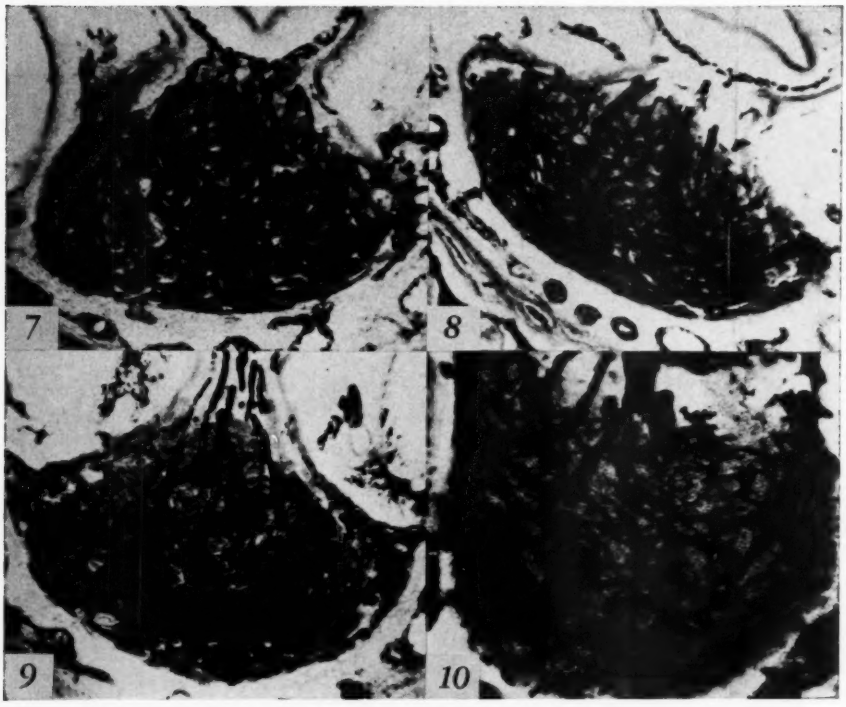

PLATE II.

Fig. 7. X-section of the pituitary of a 12-day chick embryo. 96 ×
Fig. 8. X-section of the pituitary of a 13-day chick embryo. 96 ×
Fig. 9. X-section of the pituitary of a 14-day chick embryo. 96 ×
Fig. 10. X-section of the pituitary of an 18-day chick embryo. 96 ×

Vidal, 1954). Aronsson (1952) noted a striking increase in P.A.S. positive cells during the same period. The data reported here are also supported in part by the observations of Rahn (1939), Payne (1946) and Wilson (1952). These authors have shown that a primary histological differentiation begins on the 10th day of incubation, at which time the walls of the blood vessels within the gland are in intimate contact with the glandular cords (Grignon, 1956). Thus, the above cellular changes appear to be closely associated in time with the vascular transition within the gland.

TABLE 1

HISTOGENESIS, VASCULOGENESIS, AND FUNCTIONAL DIFFERENTIATION IN PITUITARY GLANDS OF NORMAL CHICK EMBRYOS

Incubation age in days	Histogenesis	Vasculogenesis	Functional Differentiation
5		The capillary plexus of the brain surface begins to encircle the proliferating adenohypophysis (Wingstrand, 1951). Beginning of vascular penetration of the gland (Grignon, 1956).	Melanophore dispersing action observed in hypophysectomized frogs after injection of 5-day chick pituitaries in saline suspension (Chen, Oldham and Geiling, 1940). Melanophore hormone found in greatest concentration in the pars anterior, as evidenced by response in *Anolis* (Rahn, 1941).
6	Silver positive granules appear in immature basophils (Wilson, 1952). Strongly silver positive cells appear in the adenohypophysis of the chick (Wingstrand, 1951). First P.A.S. positive cells are seen in the oral pouch (Aronsson, 1952).	The encircling capillaries become incorporated by the proliferating gland (Wingstrand, 1951). Occasional penetration of irregular blood vessels is seen (Grignon, 1956). SMALL NUMBER OF CAPILLARIES DESCEND INTO THE GLAND FROM THE PRIMARY PLEXUS, ARCH OVER AND AROUND THE GLANDULAR CORDS AND COME TOGETHER IN THE VENOUS SINUS. VESSELS ARE SINUSOIDAL, SMALL AND NOT UNIFORM IN SIZE.	Thyroid gland responds to pituitary-hypothalamic complex stimulus when all are grown *in vitro* (Tixier-Vidal, 1957).
7	Number of silver positive cells increases (Wingstrand, 1951). Number of P.A.S. positive cells increases (Aronsson, 1952).	Gland divides into cephalic and caudal lobes (Atwell, 1939). MORE CAPILLARIES EMANATE FROM THE PRIMARY PLEXUS AND PENETRATE THE CENTRAL GLANDULAR MASS, PRIMARILY IN THE CEPHALIC REGION, FORMING A SMALL IRREGULAR PLEXUS. SINUSOIDS (WITHIN THE GLAND) EMPTY INTO THE EXPANDING VENOUS SINUS.	

TABLE 1 (*continued*)

Incubation age in days	Histogenesis	Vasculogenesis	Functional Differentiation
8	Basophilic tendency of cells in the pars distalis regarded as transitional (Rahn, 1939). Differentiation of the first basophils (Mosier, 1955; Wilson, 1952). P.A.S. positive cells are present in large number and are well differentiated in cephalic lobe (Aronsson, 1952).	More vessels penetrate the anterior lobe of the pars distalis than any other portion of the gland (Grignon, 1956). THE NUMBER OF ANASTOMOSING SINUSOIDS INCREASES AS THE SIZE OF THE GLAND INCREASES. THE VESSELS RETAIN THEIR IRREGULAR SHAPE. MANY ARE SEEN FLOWING INTO THE VENOUS SINUS NOW CIRCUMSCRIBING THE GLAND.	Hyperglycemia begins in hypophysectomized chick embryos (Konigsberg, 1954).
9		Separation of the gland from the base of the brain is instrumental in the establishment of portal system going into the gland (Wingstrand, 1951; Grignon, 1956). The vascular system of the adenohypophysis is practically established in 9-day embryos (Wingstrand, 1951). PORTAL VESSELS FROM THE PRIMARY PLEXUS PENETRATE THE MAIN SUBSTANCE OF THE GLAND, SPREADING TO ALL PARTS. PORTAL VESSELS ARE LARGER THAN THE OTHER SINUSOIDS. SINUSOID PLEXUS IS LITTLE INTENSIFIED.	
10	Acidophils first appear (Rahn, 1939; Payne, 1942). Acidophils first appear in the cephalic portion of the gland (Wilson,	From this day the walls of the blood vessels are in intimate contact with the glandular cords (Grignon, 1956).	Chorio-allantoic grafts of 10-day pituitary rudiments show T.S.H. activity (Studitskii, 1946). Thyrotrophic hormone level of blood at-

TABLE 1 (*continued*)

Incubation age in days	Histogenesis	Vasculogenesis	Functional Differentiation
	1952). First definite basophils are seen (Rahn, 1939). The cytoplasm of P.A.S. positive cells increases in cephalic lobe. P.A.S. positive cells are first seen in caudal lobe (Aronsson, 1952).	VESSELS WITHIN THE MASS OF THE GLAND BEGIN TO ENLARGE. VASCULAR PLEXUS WITHIN THE GLAND IS MORE INTENSE.	tains threshold of sensitivity of early thyroid (Tixier-Vidal, 1956).
11	First distinct basophils and acidophils in the cephalic lobe of the gland (Wingstrand, 1951). Decided increase in the number of P.A.S. positive cells (Aronsson, 1952). Accumulation of azan stain by some cells in the cephalic lobe. Presence of P.A.S. and P.A.F. positive cells (Tixier-Vidal, 1954).	A TRANSITION PERIOD IS EVIDENT. SINUSOIDS SHOW INCREASE IN SIZE AS WELL AS IN NUMBER. THE RESULT IS A RICH VASCULAR PLEXUS, WHICH IS IN STRIKING CONTRAST TO THE PREVIOUS DAY.	Hyphophysectomy experiments (Fugo, 1940) and chorioallantoic transplants (Martindale, 1941) indicate initial production of T.S.H.
12		VASCULAR PEAK IS REACHED. TOTAL VOLUME OF SINUSOIDS APPROXIMATES THE VOLUME OF GLANDULAR TISSUE. AN INTIMATE CONTACT IS ESTABLISHED BETWEEN BLOOD VESSELS AND CELLULAR CORDS.	
13	Existence of acidophils is confirmed (Tixier-Vidal, 1954).	DEFINITIVE VASCULAR PATTERN APPARENT. FURTHER GROWTH OF GLAND CHANGES VOLUME RELATIONSHIP IN FAVOR OF GLANDULAR TISSUE. NO FURTHER MAJOR CHANGE IN VASCULAR PATTERN.	Accumulation of adrenal ascorbic acid is first retarded in hypophysectomized chick embryos (Case, 1952). Normal weight increase in adrenals is arrested in hypophysectomized chick embryos (Case, 1952). Reduction of the ovarian cortex and intertubular tissue of testis is observed in hypophysectomized chicks, along with a reduction in size of the gonads (Fugo, 1940).

TABLE 1 (*continued*)

Incubation age in days	Histogenesis	Vasculogenesis	Functional Differentiation
14			
15	Acidophils differentiate in the caudal lobe of the adenohypophysis (Wingstrand, 1951). Appearance of cyanophils (basophils) in cephalic lobe (Tixier-Vidal, 1954).		
16	Differentiation of the basophils in the cephalic lobe (Wilson, 1952). Droplets of P.A.S positive colloid present between cells of the adenohypophysis (Aronsson, 1952).		First stimulation of the adrenal cortex, genital ducts and ovaries of prepuberal guinea pigs by injection of 16-18 day chick pituitaries (Moszkowska, 1949).
17	Intensely P.A.S. positive cells are seen in the adenohypophysis. No acidophil differentiation is evident (Mosier, 1955).		
18	Differentiation of caudal acidophils (Wilson, 1952).		

The present observations on the vasculogenesis of the adenohypophysis do not necessarily contradict the results of others which seemingly cannot be correlaed with the observed changes in vascular pattern (Table 1). Available evidence indicates that the pituitary glands of anuran and chick embryos actively synthesize and secrete hormones in small quantities at a very early stage of development. Such quantities may be insufficient to exert an effect via the blood stream. Etkin (1939) demonstrated in anurans that a trophic effect was exerted upon the thyroid primordium when it was transplanted in juxtaposition to the developing pituitary. This trophic effect upon the transplant was exerted at an earlier time than can be demonstrated in normal embryos. *In vitro* studies of pituitary-thyroid explants of developing chick embryos gave evidence for thyrotrophic activity in 6-day pituitaries (Tixier-Vidal, 1957). This is in contrast to *in vivo* experiments in which hypophysectomy (Fugo, 1940), chorio-allantoic transplants of thyroids (Martindale, 1941) and of pituitaries (Studitskii, 1946) established the beginnings of the pituitary-thyroid axis at 10–11 days of incubation. Similarly, melanophore dispersing action was noted in the chick pituitary as early as the 5th day of incubation (Chen, Oldham and Geiling, 1940; Rahn, 1941), although no histological differentiation is observable on this day. However, Wingstrand (1951) demonstrated silver positive cells in the adenohypophysis of chick embryos as early as 5 days 6 hours of incubation.

It is evident, however, that a greater degree of functional differentiation occurs around day 11. The available data suggest an early synthesis of hormones by the pituitary with release of small amounts of trophic substances during the period when distribution by means of the vascular route is inefficient. Physiological blood levels of trophic hormones seemingly are not reached before the 10th or 11th day of incubation (Fugo, 1940; Martindale, 1941; Tixier-Vidal, 1956; Thommes, 1958), when the vascular bed within the pituitary reaches a state of development which is more conducive to an efficient transport of raw materials and trophic principles.

Summary

1. Vasculogenesis in the anterior glandular portion of the pituitary gland or adenohypophysis of normal chick embryos has been studied with the aid of celloidin serial sections of embryos injected with India ink.

2. A small irregular vascular plexus forms within the gland on day 7 of incubation. These vessels originate from a primary plexus on the ventral surface of the diencephalon. This original sinusoidal pattern increases through the 9th day of incubation. On day 10 there is the beginning of a progressive change from a scant sinusoidal plexus into a complex sinusoidal pattern characterized by numerous, large, irregular vessels. This change is complete by the 12th day of incubation.

3. Changes in vascular pattern are closely associated in time with the appearance of basophils and acidophils in the cephalic lobe of the adenohypophysis as well as with P.A.S., P.A.F. and azan positive cells. The heightened vascularity corresponds in time with increased histogenesis within the gland.

4. Attainment of the definitive vascular pattern can be correlated with the initial secretion of physiological levels of T.S.H.

5. The vascular transition within the developing chick pituitary which occurs between day 10 and day 12 of incubation has been interpreted as being the anatomical prerequisite for an efficient transport of raw materials and trophic principles.

References

1. Aronsson, J. 1952. Studies on the cell differentiation in the anterior pituitary of the chick embryo by means of the P.A.S. reaction. *Kungl. Fysiograf. Sallsk Handlung.* N.F., **63**, 3-20.
2. Atwell, W. J. 1939. Morphogenesis of the hypophysis cerebri of domestic fowl during the 2nd and 3rd weeks of incubation. *Anat. Rec.*, **73**, 57-71.
3. Case, J. F. 1952. Adrenal cortical-anterior pituitary relationship during embryonic life. *Ann. N.Y. Acad. Sci.*, **55**, 147-158.
4. Chen, G., Oldham, F. K., & Geiling, E. M. K. 1940. Appearance of the melanophore-expanding hormone of the pituitary gland in developing chick embryo. *Proc. Soc. Exp. Biol. & Med.*, **45**, 810-813.
5. Dawson, A. B. 1947. Selective impregnation of cells of the basophilic series of the anterior pituitary gland of the rat and chicken: their early recognition in the fetal rat and embryonic chicken. *Anat. Rec.*, **97**, 413.
6. Etkin, W. 1939. A thyrotrophic field effect in the tadpole. *J. Exp. Zool.*, **82**, 463-495.
7. Feeney, J. F. 1944. The rapid preparation of celloidin serial sections following India ink injections. *Stain Tech.*, **19**, 137-140.
8. Flint, J. M. 1900. The blood-vessels, angiogenesis, organogenesis, reticulum and histology of the adrenal. *Johns Hopkins Hospital Rept.*, **9**, 153-229.
9. Fugo, N. W. 1940. Effects of hypophysectomy in the chick embryo. *J. Exp. Zool.*, **85**, 271-297.
10. Gersh, I., & Grollman, A. 1941. The vascular pattern of the adrenal gland of mouse and rat and its physiological response to changes in glandular activity. *Contrib. to Embryol., Carnegie Inst. of Washington*, **29**, 111-125.

11. GRIGNON, G. 1956. Développement du complexe hypothalamohypophysaire chez l'embryon de Poulet. Société d'Impressions Typographiques, Nancy, France.
12. HAMILTON, H. L. 1952. Lillie's Development of the Chick. 3rd edition. Henry Holt and Co., New York.
13. HARD, W. 1944. The origin and differentiation of the alpha and beta cells in the pancreatic islets of the rat. *Am. J. Anat.*, **75**, 369-403.
14. KONIGSBERG, I. R. 1954. The effects of early pituitary removal by "decapitation" on carbohydrate metabolism in the chick embryo. *J. Exp. Zool.*, **125**, 151-169.
15. MARTINDALE, F. M. 1941. Initiation and early development of thyrotrophic function in the incubating chick. *Anat. Rec.*, **79**, 373-393.
16. MOSIER, H. D. 1955. The development of the hypothalamoneurohypophyseal secretory system in the chick embryo. *Endocrinology*, **57**, 661-669.
17. MOSZKOWSKA, A. 1949. Pouvoir corticotrope et gonadotrope de l'hypohyse de l'embryon de Poulet. *C. R. Soc. Biol.*, **143**, 1332-1333.
18. PAYNE, F. 1942. The cytology of the anterior pituitary of the fowl. *Biol. Bull.*, **82**, 79-111.
19. RAHN, H. 1939. The development of the chick pituitary with special reference to the cellular differentiation of the pars buccalis. *J. Morph.*, **64**, 483-517.
20. ————. 1941. The distribution and development of the melanophore hormone in the pituitary of the chick. *Biol. Bull.*, **81**, 281-282.
21. STUDITSKII, A. N. 1946. Endocrine correlation in embryonic development of vertebrates. *Nature*, **157**, 427-430.
22. THOMMES, R. C. 1958. Vasculogenesis in selected endocrine glands of normal and hypophysectomized chick embryos. I. The Thyroid. *Growth*, **22**, 243-264.
23. TIXIER-VIDAL, A. 1954. Étude histophysiologique de l'hypophyse anteriéur de l'embryon de Poulet. *Arch. Anat. micr. et Morph. exp.*, **43**, 163-186.
24. ————. 1956. Étude chronologique *in vivo* et *in vitro* des corrélations hypophyse-thyroïde chez l'embryon de Poulet. *Arch. Anat. micr. et Morph. exp.*, **45**, 236-253.
25. ————. 1957. Autodifférenciation *in vitro*, sur milieu anhormonal de la fonction thyréotropé de l'hypophyse embryonnaire du poule. *C. R. Soc. Biol.*, **151**, 40-43.
26. WILLIER, B. H. 1955. Ontogeny of endocrine correlation. Analysis of Development, 574-619. W. B. Saunders Co., Philadelphia. Ed. by Willier, Weiss, and Hamburger.
27. WILSON, M. E. 1952. The embryological and cytological basis of regional patterns in the definitive epithelial hypophysis of the chick. *Am. J. Anat.*, **91**, 1-50.
28. WINGSTRAND, K. G. 1951. The Structure and Development of the Avian Pituitary. Häkan Ohlssons Boktryckeri, Lund, Sweden.

Growth, 1959, **23**, 221-233.

DIFFERENTIAL DEVELOPMENT OF THE LOIN MUSCLES IN SWINE AS AFFECTED BY AD LIBITUM AND RESTRICTED FOOD INTAKE[1,2,3,4]

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As THE animal grows, two major changes occur in the muscles; first, an increase in the size and weight and second, changes in shape and proportion to other carcass components. The effect of the plane of nutrition on growth and size is well known, although the extent of the effects are not sufficiently appreciated. In programs of selective breeding for the meat type hog, it is essential that the measures used to evaluate traits be as closely associated with the traits as possible. Random errors in trait appraisal act in the same manner as environmental variations in reducing the effective heritability of a desired trait. Since the loin is one of the most valuable carcass cuts it is important that it constitute a reasonably high proportion of the carcass. At the same time it is desirable to have an objective measure for appraising loin quality in order to promote genetic improvement.

The degree and rate of muscle development is under genetic control as judged by breed differences (Fredeen *et al.*, 1955) and the role of heterosis (Gregory and Dickerson, 1952). The interactions between nutrition and genetics in determining the composition of meat are complicated. The reason for these complications is that meat is

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produced by different parts, which are themselves differently affected by nutritive conditions so that nutritive conditions will affect one part of the body much more than another. Thus genetic influence is much greater in some cuts or specific parts of the body than in others which are under the influence of environmental conditions.

The classical studies on differential growth of the pig as affected by the plane of nutrition have been undertaken by Hammond and Murray (1937) and McMeekan (1939, 1940, 1941).

The objectives of these studies were to determine the effects of long-term controlled nutrition on the development of the meat type hog as judged by loin muscles development. The possible effects of pregnancy have been also investigated.

Materials and Methods

Experimental animals: Thirty-four Palouse gilts of four nutritional planes were used in the present experiment. The animals are of the 10th generation of a crossbreeding experiment which was initiated in 1947 as follows:

In 1947, crossbreeding started from 5 groups of relatively unrelated Chester White gilts; each group consisted of 5 females and was bred to a different Danish Landrace boar. The litter of each female, at 8 weeks of age, was divided equally and at random into two groups. One line was on full feed (self-fed). The other line was on 70 percent of full feed (as determined by a growth curve which was established by a pilot trial). After six generations, one-half of the pigs in each group were shifted to the other plane of nutrition (Table 1), thus forcing four groups; high-high (HH), low-low (LL), and low-high (LH)

TABLE 1
Experimental Groups

Experimental Group	Level of Feeding F_1 to F_6	Level of Feeding F_7 to F_{10}	Number of Animals Slaughtered	
			38 days post-coitum	100 days post-coitum
HH (High High)	Full fed*	Full fed	5	4
HL (High Low)	Full fed	Limited fed**	6	4
LH (Low High)	Limited fed	Full fed	4	3
LL (Low Low)	Limited fed	Limited fed	5	3

* Self fed.
** 70% of full feed as determined by a growth curve previously established by a pilot trial.

and high-low (HL). During the ten generations the breeding stock was selected as the pigs reached 150 lbs. on the basis of the selection index: $I = B + 2W + 35G$, where B is the number of pigs born alive, W is the number of pigs weaned in the litter in which the individual pig was born, and G is the average rate of daily gain of the individual pig from weaning to 150 lbs. This index places approximately equal emphasis on prolificacy and rate of gain based on variability. No attention was given to desirability of type. The highest indexed animals were selected.

Feeding: The composition of the rations fed to the four experimental groups was identical. The ration consisted of ground barley, ground wheat, mill run, corn distillers dried solubles, soybean meal, fish meal, dried skim milk, dehydrated alfalfa meal, ground oyster shell, steamed bone meal, iodized salt and mineral mix. Detailed information on the feeding and breeding systems has been published by Brugman (1950).

Slaughter: Thirty-four gilts representing the four planes of nutrition (HH, HL, LH, and LL) in F_{10} were checked daily with vasectomized boars and were bred 60 days after puberty. The gilts were randomly assigned to be slaughtered at 38 and 100 days after breeding. The dressed split carcass was chilled for 48 hours and weighed. The carcass was processed into the following cuts; jowl, picnic ham, Boston butt, loin, bacon and ham.

The six cuts of the right half were dissected into lean, subcutaneous fat, intermuscular fat, skin, and bone. The *Longissimus dorsi* and the *Psoas major* (tenderloin) muscles were removed intact. The dissected tissues were weighed in plastic bags to minimize the loss by evaporation. The loin of the left half was cut across the *Longissimus dorsi* at the 10th rib for surface area mesurement. *The Loin Square Measure* (Figure 1). A simple design was used to measure the width and length of the *Longissimus dorsi* cross section and the backfat thickness. The square measure was made of millimetric acetate paper or a combination of graph paper and tracing paper. The following technique was used to obtain accurate and comparable results:

a. The loin square measure was placed on the loin eye muscle with the ventral surface of the muscle parallel to the base of the square measure (B), so that the center point fell approximately on the center of the loin eye.

b. Keeping the square measure parallel to the ventral surface of

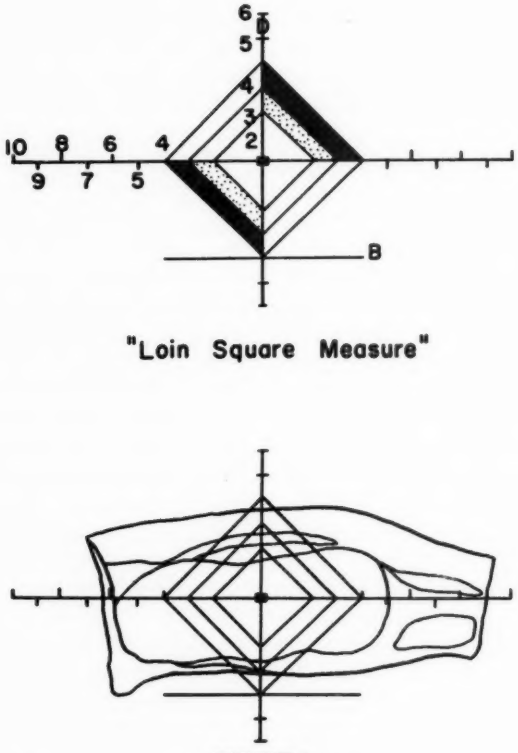

FIGURE 1

Upper: The loin square measure made of millimetric acetate paper was used to measure the width and depth of the *Longissimus dorsi* cross section. Lower: The loin square measure placed in position on the *Longissimus dorsi* muscle.

the loin muscle, the former was raised or lowered until its center point was centered vertically along the loin muscle.

c. Keeping the square measure parallel to the ventral surface of the loin muscle, the former was moved to the right or left until its center point was centered horizontally on the loin muscle.

d. The square measure was recentered vertically.

e. The width of the *Longissimus dorsi* was measured on the horizontal line. The depth of the muscle was measured on the vertical line (D).

f. The point at which the vertical line of the square measure crossed the loin muscle was marked. The backfat thickness was measured at that point.

Statistical evaluation of the results were made by the analysis of covariance (adjusted to live weight). All data were transposed to I.B.M. cards to permit simultaneous computation of all desired correlations.

RESULTS

Carcass and components: The live weight of the experimental animals was corrected to an empty live weight by subtracting the weights of the alimentary canal contents and the reproductive tract. The dressing percentages expressed to empty live weight ranged from 75.23 to 80.41. The average percentage of the wholesale loin to carcass weight ranged from 23.61 to 26.87 (Table 2). The plane of nutrition and the stage of pregnancy had no significant effect on these characteristics. The weight of the wholesale loin, and the thickness of backfat were increased in the high plane groups (Figure 2).

Development of loin muscles: In the 38-day pregnant gilts the average weight of the dissected *Longissimus dorsi* varied from 3.87 to 5.36 lbs. The average weight of the *Psoas major* varied from 0.47 to 1.20 lbs. The ratio between the weight of *Psoas major* to the weight

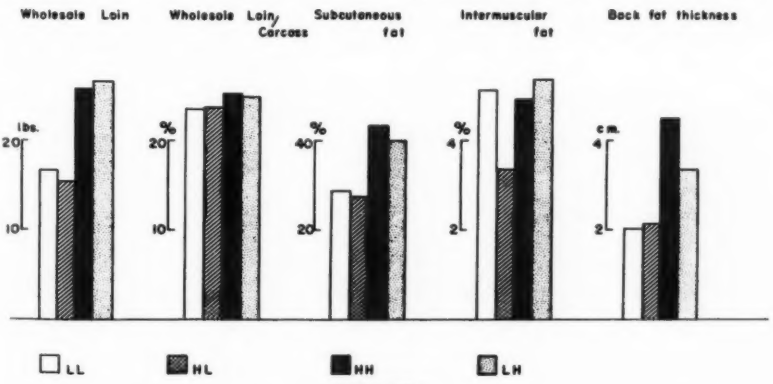

FIGURE 2

The effect of plane of nutrition on the wholesale loin and fat deposition. The plane of nutrition affected the weights of wholesale loin and subcutaneous fat and backfat thickness but not the percentage of wholesale loin to carcass.

TABLE 2
EFFECT OF FEEDING LEVEL ON CARCASS AND LOIN CUT
(Averages of Experimental Groups)

Nutritional Group	Stage of Pregnancy (days)	Empty Live Wt.[a] (lbs.)	Wt. of Wholesale Loin (lbs.)	Dressing %[b]	Wt. of Wholesale Loin to Carcass Wt. (%)	Backfat (cm.)
LL	38	194.58	16.51	75.60	23.61	2.0
	100	287.09	26.99	80.26	23.98	3.3
HL	38	181.22	15.56	75.23	23.81	2.1
	100	263.61	23.41	76.08	24.67	3.0
HH	38	274.77	25.93	79.12	25.11	4.5
	100	318.63	34.57	80.41	26.32	4.8
LH	38	286.67	26.79	78.90	24.91	3.6
	100	392.49	41.81	72.19	26.87	6.3

[a] Live weight after 24 hours fasting (weight of alimentary canal content + weight of genital organs with fetuses).
[b] This was based on the weight of dressed carcass with head, feet, kidneys and leaf fat after cooling for 48 hours.

TABLE 3
EFFECT OF FEEDING LEVEL ON DEVELOPMENT OF *Longissimus Dorsi*
(Averages of Experimental Groups)

Nutritional Group	Stage of Pregnancy (days)	Wt. of L.D.[a]	Wt. of L.D.[b] / Wt. of Loin Lean %	L.D. Surface area[c] (sq. in.)	L.D. depth: ratio (1: —)	Wt. of P.M. (lb.)	Ratio of wt. of P.M.:L.D. (1: —)
LL	38	4.28	48.79	4.98	2.31	0.80	5.35
	100	6.17	53.96	6.19	2.04	1.03	6.17
HL	38	3.87	51.13	4.49	1.93	0.77	6.53
	100	5.08	53.95	5.10	2.04	0.94	6.70
HH	38	5.36	54.39	5.26	1.77	0.80	6.78
	100	6.12	50.92	5.73	2.06	0.89	6.53
LH	38	5.12	51.19	5.46	2.01	1.06	5.82
	100	6.59	50.93	5.54	2.05	1.06	6.22

L.D. = *Longissimus dorsi*
P.M. = *Psoas major*
[a] This only included the weight of *Longissimus dorsi.*
[b] This excluded the tenderloin muscle.
[c] Measurements were taken from a cross section of the *Longissimus dorsi* at the 10th rib.

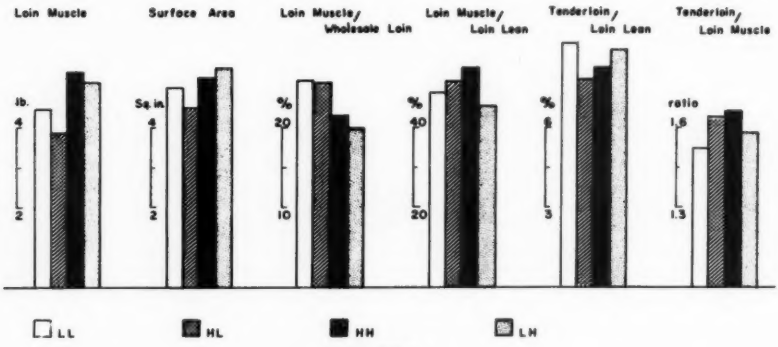

FIGURE 3

The effect of plane of nutrition of differential growth of *Longissimus dorsi* and *Psoas major* muscles in relation to fat deposition.

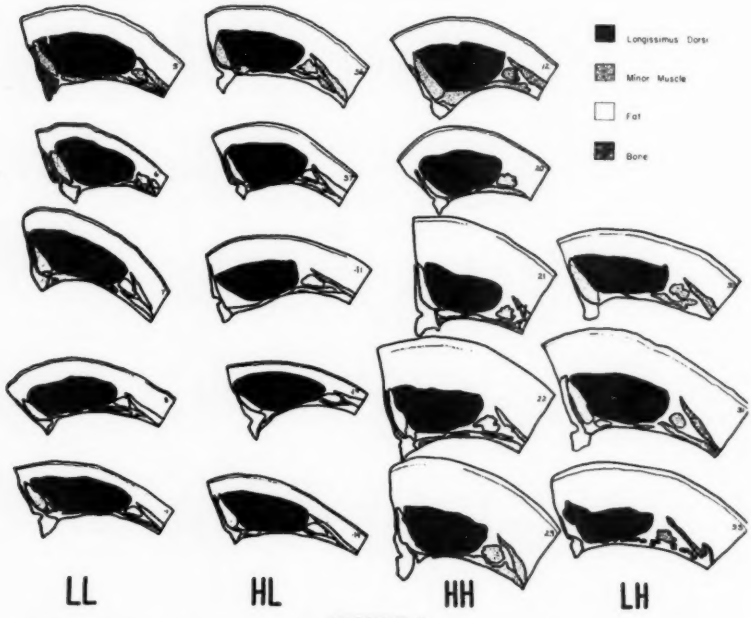

FIGURE 4

The tracings of pork chops of the four experimental nutritional groups. The individual differences in *Longissimus dorsi* cross section area were larger than group differences. Note the differences in the shape of the muscle.

of the dissected *Longissimus dorsi* ranged from 1:5.35 to 1:6.78. The average percentage of the dissected *Longissimus dorsi* to the total loin lean varied from 48.79 to 54.39 (Table 3, Figure 3).

The average surface area of the cross sections of the *Longissimus dorsi* at the 10th rib varied from 4.49 to 5.46 sq. in. (Figure 4). The depth:length ratio of the *Longissimus dorsi* cross section at the 10th rib was measured by the "loin square meter." This ratio varied from 1:5.35 to 1:6.78. There were no significant differences between the high and the low planes of nutrition as indicated by the analysis of covariance (after adjustment for empty live weight). Also there were no significant differences between LL and HL groups or between HH and LH groups.

In the 100-day pregnant gilts muscle development was significantly higher than in the 38-day pregnancy. It would appear that such differences were due to the function of live weight and age rather than the effect of pregnancy *per se*.

Correlation Studies: The values of all the experimental pigs were adjusted; live weight and correlation were computed. The surface area of the *Longissimus dorsi* cross section was positively correlated with each of the following values; weight of this muscle, weight of total lean in the loin cut and weight of total lean in half the carcass (Table 4, Figure 5). The surface area, however, was not related to dressing percentage, weight of tenderloin muscle and $\frac{\text{length}}{\text{depth}}$ of *Longissimus dorsi* cross section. This may indicate that the factors governing the development of *Longissimus dorsi* in length are different from those governing the development of plumpness. It may also indicate that the rate of development of the two major muscles of the loin is not identical.

TABLE 4
CORRELATIONS OF SURFACE AREA OF *Longissimus Dorsi* WITH MUSCLING CHARACTERISTICS

Correlated values	r = Non-significant	r = Significant
Surface area of L.D. × dressing %	0.1498	
Surface area of L.D. × $\frac{\text{depth}}{\text{width}}$ L.D.	0.1702	
Surface area of L.D. × total lean in ½ carcass		0.6825*
Surface area of L.D. × weight of L.D.		0.8376*
Surface area of L.D. × weight of Psoas major	0.4249	
Surface area of L.D. × weight of lean in loin		0.6162**

* Significant at 1% level.
** Significant at 5% level.
n = 34.

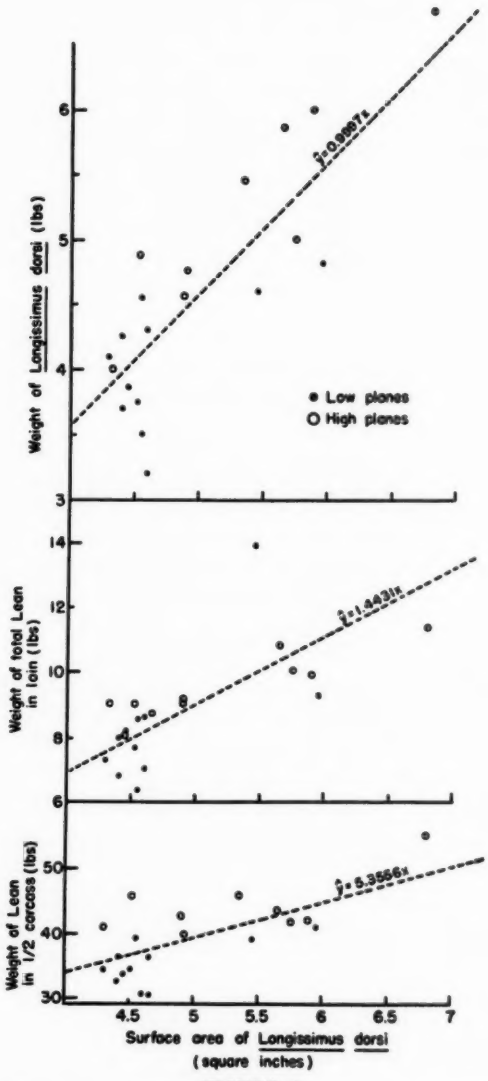
Weight of Longissimus dorsi (lbs)
Low planes
High planes
Weight of total Lean in loin (lbs)
Weight of Lean in 1/2 carcass (lbs)
Surface area of Longissimus dorsi (square inches)

FIGURE 5

Correlation between *Longissimus dorsi* cross section surface area and muscling properties of the carcass. Note the degree of correlation.

It is the shape rather than the cross-section area of the muscle which determines its suitability for the high quality trade. Thus, the width of the *Longissimus dorsi* minus its depth, gave a correlation of —0.7221 with carcass total lean. By taking the total length of the carcass into account (from the aitch bone to the first rib) on the basis that the total muscle development is related to the linear as well as cross section surface of the muscle, a similar high "r" value was obtained.

Discussion

The results show that long term controlled level of feeding did not accelerate muscle development. The individual variations within the same plane of nutrition in the same breed are much higher than previously anticipated. In the high plane groups of nutrition, the later developing tissues of the body, such as fat, are speeded up in development, whereas in the low plane groups the earlier developing tissues such as the bones grow at the expense of such later developing parts as fat.

It appears that the muscling properties of the carcass are under the control of complex genetic factors. Willman and Krider (1943) reported little association between fatness and area of loin eye muscle, or the lean area in the butt end of the ham.

In the young pigs, the *Longissimus dorsi* muscle is thin in relation to the length of the spinal process of the vertebrae; the muscle thickens in proportion as the animal grows. It has been reported that the surface area of the *Longissimus dorsi* is significantly correlated with the live probe (Pearson *et al.*, 1957), backfat thickness (Hazel and Kline, 1952), the percent of lean in ham, the percent weight in the proximal portion of the ham (Fredeen *et al.*, 1955) and the percent of lean cut of loin and ham (Whiteman and Whatley, 1953). Moreover, Zobrisky *et al.* (1954) have demonstrated that the loin eye cross section area is influenced to a greater extent than the ham lean cross-section area per unit change in carcass length. Their results can be rationalized as follows: With any given amount of muscle, an increase in length is at the expense of the cross section area or, conversely, an increase in cross section area is at the expense of length.

The results indicate that the development of the *Longissimus dorsi* cross section area is not associated with the development of the *Psoas major* muscle. Zobrisky *et al.* (1954), reported that the cross

section area of the *Longissimus dorsi* and the tenderloin are positively correlated with the yield of lean, r = 0.60 and 0.19, respectively. In a study of four ham muscles, Hiner and Hankins (1939) found that these muscles did not consistently change in weight with an increase in ham plumpness. They found a positive correlation between separable fat and ham plumpness and a negative "r" between separable lean and ham plumpness. The "r" value between separable fat and separable lean was negative and very high.

Several methods have been used for carcass evaluation. Examples include the live probe, specific gravity, antipyrine solubility in body water, blood volume, urine creatinine and X-ray. Systems of scoring which depend on visual evaluation of loin quality are not so accurate nor so widely adaptable as would seem to be necessary to contribute consistently in a selective breeding program. It is suggested that the inclusion of a measure of loin leanness in routine carcass evaluation would serve two purposes: provide an objective measure of loin, and place greater emphasis on the quantity of lean in the entire carcass.

Summary

Palouse gilts were divided into two groups; one group was full fed from weaning; the other group was fed only 70% this amount. After six generations, one-half of the pigs in each group were shifted to the other plane of nutrition, thus forming four groups: high-high (HH), low-low (LL), low-high (LH), and high-low (HL). Thirty-four gilts of the 10th generation were slaughtered. The carcasses were processed to wholesale cuts. The right half of each carcass as dissected to lean, subcutaneous fat, intermuscular fat, bone and skin. The dissected *Longissimus dorsi* and *P. major* in muscles were weighed. The surface area of the *Longissimus dorsi* cross section was measured by planimetry and the width and depth were meaured by a "loin square measure." The plane of nutrition had no significant effect on the corrected empty dressing percentage or the percentage of wholesale loin to carcass. The weights of wholesale loin, and the subcutaneous fat and the thickness of backfat were affected by plane of nutrition. The weight of the dissected *Longissimus dorsi* varied from 3.21 to 6.72 lbs. and the *Psoas major* from 0.47 to 1.20 lbs.; these were not affected by the plane of nutrition. Individual differences in the surface area of *Longissimus dorsi* cross section were larger than group differences; this denotes a genetic background. This surface area was

positively correlated with the weight of the same muscle, weight of total lean in loin and in carcass. The surface area was not related to dressing percent, nor weight of tenderloin. The width of the *Longissimus dorsi* cross section minus the depth gave a negative correlation. The results are discussed in relation to patterns of differential growth and to methods of carcass evaluation.

References

Brugman, H. H. 1950. The effect of the plane of nutrition on the carcass quality of a line of swine based on a Chester White and Danish Landrace cross. *Journal of Animal Science,* **9**, 602.

Fredden, H. T., Bowman, G. H., & Stothart, J. G. 1955. Relationships between certain measurements of ham and carcass quality. *Canad. J. Agric. Sci.,* **35**, 95.

Gregory, K. E., & Dickerson, G. E. 1952. Influences of heterosis and plane of nutrition on rate and economy of gains, digestion and carcass composition of pigs. *Mo. Agric. Exp. Sta. Res. Bul.* No. 493.

Hammond, J., & Murray, G. N. 1937. The body proportions of different breeds of bacon hogs. *J. Agric. Sci.,* **27**, 394.

Hazel, L. N., & Kline, E. A. 1952. Mechanical measurement of fatness and carcass value on live hogs. *Journal of Animal Science,* **11**, 313.

Hiner, R. L., & Hankins, O. G. 1939. Significance of variation in ham conformation. *J. Agric. Res.,* **39**, 293.

McMeekan, C. P. 1939. The nature and variability of the carcass characters of Danish and English bacon pigs. *J. Agric. Sci.,* **29**, 131.

———. 1940. Growth and development in the pig, with special reference to carcass quality characters, **30**, 276.

———. 1941. Growth and development in the pig with special reference to carcass quality characters. *J. Agric. Sci.,* **31**, 1.

Pearson, A. M., Price, J. F., Hoefer, J. A., Bratzler, L. J., & Magee, W. T. 1957. A comparison of the live probe and lean meter for predicting various carcass measurements. *Journal of Animal Science.* **12**, 591.

Whiteman, J. V., & Whatley, J. A. 1953. An evaluation of some swine carcass measurements. *Journal of Animal Science,* **12**, 591.

Willman, J. P., & Krider, J. L. 1943. A study of the characteristics of live market hogs as related to the quality of carcass produced. *Journal of Animal Science.,* **2**, 231.

Zobrisky, S. E., Lasley, J. F., Brady, D. E., & Weaver, L. A. 1954. Pork carcass evaluation. *Mo. Agric. Expr. Sta. Res. Bul.* No. 554.

Growth, 1959, **23**, 235-249.

DIFFERENTIATION OF THE GLYCOGEN BODY OF THE CHICK EMBRYO UNDER NORMAL AND EXPERIMENTAL CONDITIONS

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INTRODUCTION

ONE of the primary differences between the avian spinal cord and that of other vertebrates is the presence in the former of a glycogen body which is located between the dorsal halves of the nerve cord at the level of the lumbo-sacral plexus (Imhof 1905, Terni 1924, Watterson 1949). This peculiar structure arises in the chick embryo at about 8 days of incubation and accumulates glycogen throughout the remainder of development until some unknown time in the adult (Terni 1924, Doyle and Watterson 1949). The function of the glycogen body is unknown.

Conflicting views are found in the literature concerning the histology of the glycogen body (Imhof 1905, Terni 1924, Watterson 1949). Moreover, nothing is known concerning the factors or conditions which bring about the differentiation of the glycogen body and the accumulation of glycogen by that structure (Watterson *et al.* 1958). The purpose of this study was to examine the differentiation and basic histology of the glycogen body under normal developmental, chorio-allantoic and *in vitro* conditions using histological and cytochemical techniques. It was believed that something could be learned about the nature of the conditions which govern the differentiation of the glycogen body if presumptive glycogen-body tissue were isolated from the embryo and grown on the chorio-allantoic membranes and also cultured *in vitro*. Evidence will be presented to show that the cells of the glycogen body possess an innate capacity for synthesis of glycogen.

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Materials and Methods

White leghorn eggs were incubated in a forced-draft incubator at a temperature of 37.5°C. and a relative humidity of 50-60%.

Differentiation of the glycogen body of the chick embryo was studied under the following conditions. (a) normal development; (b) chorio-allantoic grafting, and (c) cell culture *in vitro*.

Procedures used in the study of normal development

The development of the glycogen body was studied in tissue sections taken from chick embryos at daily intervals from 7 through 19 days of incubation and from newly-hatched chicks. The lumbo-sacral region of the embryo was removed and fixed in 9 parts absolute alcohol and 1 part neutral formalin for 18-24 hours at 5°C. Fixed tissues were embedded in paraffin and cut in serial sections at 8 micra. Several techniques of staining were used to clarify the morphology of the cells making up the glycogen body. These were as follows: (A) Some sections were stained with hematoxylin and eosin for general histological study. (B) The presence of polysaccharides was detected by the periodic acid-Schiff's reaction (PAS), (McManus 1948). As a control for glycogen, alternate sections were incubated in 0.1% malt diastase in phosphate buffer of pH 7.0 for 1 hour at 38°C. Some sections were incubated in saliva in order to check the specificity of the malt diastase preparation for glycogen. These sections were then subjected to PAS along with the unincubated sections. (C) Nuclei were stained with Feulgen reagent according to the procedure recommended by Gomori (1952) for the detection of DNA. (D) In some sections, cytoplasm was stained with toluidine blue in sodium acetate buffer of pH 4.2 for 3 hours to detect RNA (Gelfant *et al.* 1955). Some sections were pretreated in 10% perchloric acid for 18 hours at 5°C. to extract RNA and serve as controls. (E) Nervous tissue was stained with activated protargol according to the method of Bodian (1937) as modified by Ungewitter (1943).

Procedures used in chorio-allantoic grafting

The method of chorio-allantoic grafting was essentially the same as that described by Hoadley (1924). The hosts consisted of White Leghorn chick embryos incubated for 8-9 days.

Donor embryos were removed from the egg and placed in sterile petri dishes. Presumptive lumbo-sacral tissue and nerve cord was

isolated from 7-8 day, 3-4 day and 33-48 hour chick embryos as follows: The developing tissue of the sacral region of 7-8 day embryos (stages 30-34 of Hamburger and Hamilton 1951) was removed by transection of the embryo at the level of the twenty-fifth somite. The posterior limb-buds were cut off close to the vertebral column and discarded. The sacral region was trimmed on all sides to the outer limits of the vertebral cartilage and then cut into smaller pieces (about 2-3 mm. thick) in a small amount of warm Ringer's solution (Ringer-Locke). The presumptive sacral tissue of 3-4 day embryos (stages 19-23) was isolated by removing the tail-bud at the twenty-fifth somite. The limb-buds were discarded and the tissue was cut into smaller pieces in Ringer's solution. The presumptive tissue of the future sacral region of 33-48 hour embryos (stages 11-13) was isolated by removing the caudal portion of the embryo just below the level of the last-formed somite so as to include the posterior primitive streak and the sinus rhomboidalis of the neural tube. These were placed in Ringer's solution and left intact. Presumptive cervical tissue and nerve cord was isolated and prepared in the same manner to serve as controls. These were taken just above the level of the anterior limb-buds in 7-8 day and 3-4 day embryos, and from the fourth to the tenth somite in 33-48 hour embryos.

One piece of donor tissue was transplanted to the chorio-allantoic membrane of a host embryo using watchmaker's forceps. Grafts were recovered at 18 days of incubation. These were fixed in absolute alcohol-formalin for 12-24 hours, cut in paraffin sections at 8-10 micra and stained with PAS to identify the glycogen body. Some sections were incubated with malt diastase and some with saliva to serve as controls.

Procedures used in cell culture

Cell suspensions were prepared from the lumbo-sacral region of the nerve cords of 6, 8, 12 and 14-day chick embryos and cultured for 12 days in 200 ml. glass prescription bottles. The following procedure was used: After 8-12 embryos had been removed from eggs and placed in sterile petri dishes, the lumbo-sacral regions of their spinal column were isolated and transferred to a second dish containing a small amount of Ringer's solution. Some pieces were immediately fixed and sectioned to determine whether glycogen body substance was present before culture. The nerve cords were removed from each piece, teased

apart in Ringer's solution and transferred to a 20 ml. test tube by means of a pipette. A mixture of 10 ml. of Ringer's solution and 0.5 ml. of reconstituted trypsin (Bacto-Trypsin, Difco) was added to the tube by means of a syringe. The tube was then placed in a water bath at 38°C. for 20 minutes and shaken at 5 minute intervals. At the end of that time, the cells were prepared for culture by centrifugation and washing in the same manner as described by Wright and Sagik (1958) for chick embryo kidney cells.

The number of cells per ml. was determined by mixing an aliquot of the suspension with neutral red (1 part neutral red to 1000 parts Ringer's) and counting the stained cells in a standard hemacytometer. To determine the number of glycogen-storing cells, another portion of the suspension was centrifuged at 1000 rpm for 5 minutes and fixed in absolute alcohol-formalin for 5 minutes. After fixation, the cells were washed and centrifuged in 70% alcohol, 40% alcohol and distilled water. The cells were then mixed with 0.5% periodic acid for 10 minutes. This suspension was centrifuged and the supernatant was discarded. Schiff's reagent was added in an amount to restore the original volume of the sample. After 20 minutes, the number of cells that were stained with the PAS reaction were counted using the same hemacytometer as before.

An inoculum containing 2.3-5.0 x 10^6 cells per ml. was introduced into each bottle. Bottles were fitted with neoprene stoppers and incubated at 37.5°C. with the flat side down. The culture media consisted of 0.5% lactalbumin hydrolysate in Hank's solution, 20% calf serum, 2.0 mM. L-glutamine and the vitamin mixture recommended by Eagle (1955). In addition, each ml. of media contained 200 units of penicillin, 200 μgm. of streptomycin and 200 units of Mycostatin (Squibb). Phenol red was used as an indicator of pH. Every 3-4 days, the used medium was replaced with fresh medium of the same composition as that given at the start of culture except that Earle's solution was substituted for Hank's. A total of 39 cultures of lumbo-sacral nerve cord were kept for 12 days along with 5 cultures of cervical nerve cord as controls. At the end of that time, each bottle was emptied of media and rinsed with Ringer's solution. Two cultures of each series were fixed with alcohol-formalin, rinsed with distilled water and filled with 20 ml. of 0.5% periodic acid. After 10 minutes, the bottles were emptied, rinsed with water and filled with Schiff's reagent. Each bottle was examined under the microscope for the presence of PAS-

positive cells. As a control for glycogen, some bottles were incubated with diastase or saliva before treatment with PAS. In at least 4 of the remaining cultures of each series, cells were isolated from the bottles with trypsin-Ringer's solution. These cells were centrifuged, washed, stained and counted in the same manner as was done before culture in order to ascertain whether there was an increase in the total number of cells and of the glycogen-storing cells.

Results

Normal Development

Using the PAS-reaction, the first indication of the glycogen body in the chick embryo was found between 7-8 days of incubation. At that time, small granules of PAS-positive material could be seen in the periphery of some cells that comprised the medial and lateral parts of the roof plate in the lumbo-sacral region of the nerve cord (Figure 1). These granules were not seen after similar sections were incubated with malt diastase or saliva previous to treatment with PAS. These cells were oval in shape and measured about 4 x 6 micra in an embryo of 8 days. There was a rapid increase in the amount of PAS-positive material in the roof plate during the next 24-48 hours, so that by 10 days, a channel of glycogen-storing cells was seen extending from the neural canal to the dorsal limits of the nerve cord. The mean size of 25 cells was found to be 6.0 x 8.1 micra at that time of incubation. At 12 days, the glycogen body appeared as a large wedge of tissue which was bounded laterally by the dorsal funiculi of the nerve cord (Figure 3). The size of the cell was found to be 8.3 x 12.5 micra at 12-13 days of incubation (Figure 2). Many mitotic figures were noted throughout that area when similar sections were treated with Feulgen reagent.

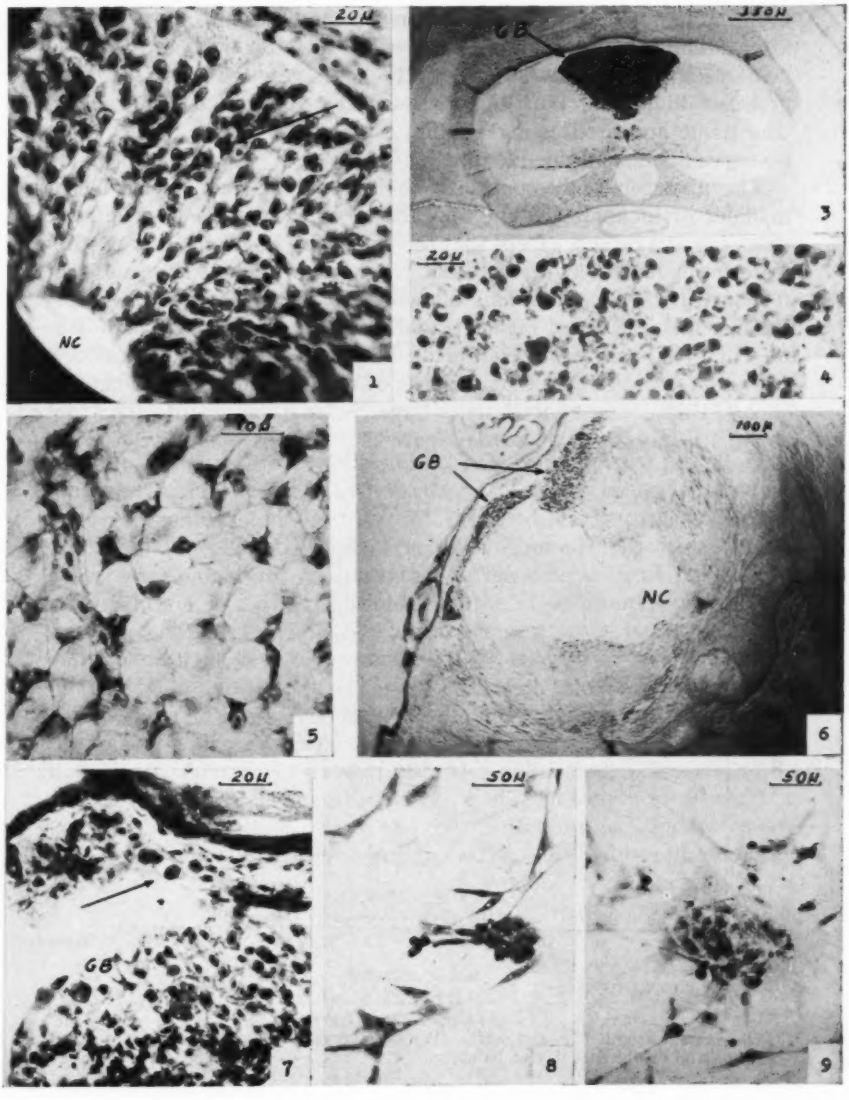
The amount of glycogen was so extensive at 12 days that is was difficult to discern the outline of the cells. A study of PAS-treated sections revealed only the presence of large granular formations which obscured any cell boundaries (Figure 4). The majority of cells appeared polyhedral in form after staining with hematoxylin and eosin. The nucleus was spherical and was often found at the edge of the cell. Only a small amount of material surrounding the nucleus was stained with eosin or toluidine blue. In most cases, this small margin of material around the nucleus tapered sharply toward the cell mem-

PLATE I

Explanation of Figures

1. Cross section through the roof plate of the lumbo-sacral region of the nerve cord at 7-8 days of incubation. The arrow points to a cell of the beginning glycogen body. PAS and hematoxylin.
3. Cross section through the spinal column showing the nerve cord and glycogen body at 12-13 days of incubation. PAS.
4. Higher magnification of the glycogen body in figure 3. The large granules represent glycogen. PAS.
5. Cells of the glycogen body at 13 days of incubation. Toluidine blue.
6. Section through a 10-day chorio-allantoic graft of a 48 hour donor. The arrows point to glycogen body tissue in the graft. PAS.
7. Higher magnification of the glycogen body in Figure 6. The arrow points to a cell of the glycogen body. PAS and hematoxylin.
8. A cluster of glycogen body cells in a culture of 8-day lumbo-sacral nerve cord. PAS and hematoxylin.
9. Glycogen body cells in a culture of 12-day lumbo-sacral nerve cord. PAS and hematoxylin.

NC neural canal
GB glycogen body


20H
NC
1
GB
330H
3
20H
4
70B
5
GB
100R
NC
6
20H
GB
7
50H
8
50H
9

brane (Figure 5). This material showed the presence of red granules after staining with toluidine blue at pH of 4.2 for 3 hours. Pretreatment with cold perchloric acid for 18 hours resulted in a loss of red granules indicating that this perinuclear material contained RNA. The tissue appeared as a reticulum after staining with protargol but no nerve fibers could be identified.

The glycogen body continued to increase in size during the remainder of incubation. The appearance of the tissue was similar to that seen at 12 days, except that the cells were larger in size and there were fewer mitotic figures present. At hatching time, some cells were filled with PAS-positive material while others appeared to be empty. An examination under higher magnification revealed that small granules of PAS-positive material could be found in the majority of cells. It is interesting to note that the cells of the glycogen body at hatching time (21 days) are about 4 times larger in length and width than those found at 8 days of incubation (Figure 2).

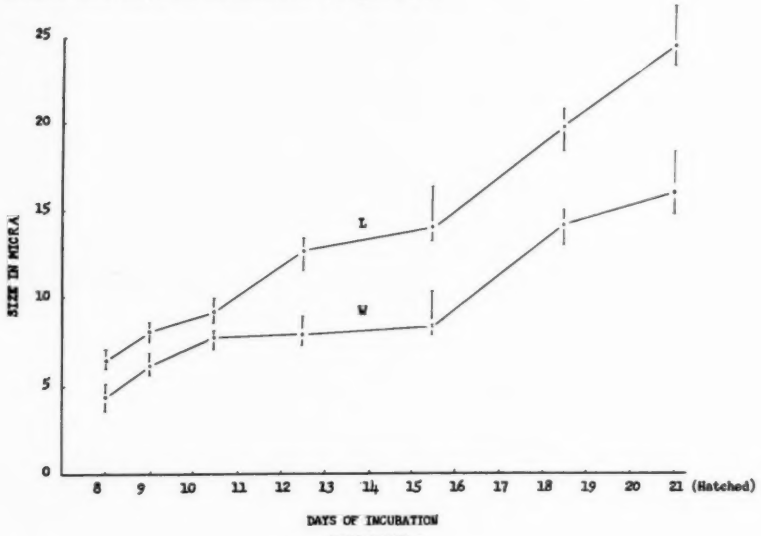


FIGURE 2

Explanation of Figure

The average length (L) and width (W) of glycogen body cells in micra during incubation and on the first day of hatching.

| · maximum variation per group of 25 cells.
|

The stored material in the glycogen body during incubation and on the first day of hatching was identified as glycogen because PAS-positive material was not found when sections were incubated with malt diastase or saliva previous to treatment with the periodic acid-Schiff's reaction.

Chorio-allantoic Grafting

It was found that differentiation of the glycogen body could be attained when segments of the lumbo-sacral region of the spinal column were grafted to the chorio-allantoic membranes of 8-9 day chick embryos. In general, grafts which were recovered from 7-8 day donors showed a higher incidence of glycogen body than did those which were recovered from similar transplants of 3-4 day or 33-48 hour embryos (Table 1). In any case, tissue of the glycogen body could always be identified because it showed the presence of PAS-positive material in cells which were grouped together (Figure 6). The majority of cells were oval-shaped with eccentrically placed nuclei (Figure 7). Pretreatment with diastase or saliva resulted in a negative PAS-reaction which indicated that this material was glycogen. In addition, the cells of the glycogen body showed an increase in size which was comparable to that noted during normal development. The size of the cell in grafts recovered from the 33-48 group of donors with a control age of 11-12 days was the same as that found in control embryos at 9-10 days of incubation. Glycogen body cells from grafts of the 3-4 day donors corresponded in size to that found in a control age of about 12 days. The largest cells were found in grafts recovered from the 7-8 day group of donors; these were comparable in size to that found about 16-17 days of normal development. The cells of the glycogen body in these grafts attained a size indicative of an age that was 1-2 days less than that of the control as shown by measurements of the glycogen-storing cells. One to two days probably represents the

TABLE 1
CHORIO-ALLANTOIC TRANSPLANTS AND GRAFTS

Donor Age	7-8 days	3-4 days	33-48 hours	Total Number Lumbo-sacral	Cervical (control)
Transplants	19	33	11	63	22
Grafts	10	19	8	37	12
Grafts with glycogen body	8	12	4	24	0
% Grafts with glycogen body	80	63	50	65	0

period of time during which the transplant was incorporated into the chorio-allantoic membrane of the host.

Cell Culture

Groups of cells were seen to adhere to the glass surface of the bottle after 3 days of culture. It was not possible to make detailed observations concerning the behavior of isolated cells before this time because of the thickness of the bottle and the lack of temperature control on the microscope stage. Cultures of 12- and 14-day lumbo-sacral nerve cord seemed to proliferate more rapidly than did those of 6- or 8-day donors.

After fixation with alcohol-formalin and treatment with the PAS-reaction, it was found that the cells remained attached to the glass surface of the bottle. Granules of PAS-positive material could be seen in cells by direct microscopical observation through the culture bottle. The cells in each culture which stained with PAS were mainly oval or spherical in form and were found in clusters or in the center of a larger group of cells (Figures 8 and 9). Pretreatment with diastase or saliva resulted in a loss of the PAS-reaction in the same type cells of similar cultures. Cultures of cervical nerve cord were PAS-negative and consisted mainly of spindle-shaped cells which grew in a linear or chain-like fashion. Each culture showed an increase in the total number of cells with the greatest increase occurring in cultures from 12- and 14-day donors. These cultures also showed the greatest number of glycogen-storing cells. In cultures of 6-day lumbo-sacral nerve cord, glycogen body cells were not seen in the counting chamber or in paraffin sections before culture but both 6- and 8-day cultures of lumbo-sacral nerve cord showed glycogen-storing cells at the end of 12 days of culture.

Discussion

Histology of the Glycogen Body

Remak (1838, cited in Duval 1877, page 15), published the first microscopical study of the glycogen body. He described the tissue as consisting of small globules which were richly supplied with blood vessels. Leydig (1854) described the glycogen body as that of a reticulum of cells which were anastomosed to each other by their cytoplasmic processes. Duval (1877) felt that Leydig was wrong and that the reticular appearance of the tissue was an artifact which was

induced by the many layers of cells superimposed upon each other in a tissue section. Imhof (1905) maintained that the tissue was a reticulum of neuroglia-like cells and that the material of the glycogen body was stored between the cells of the reticulum intercellularly. Hansen-Prus (1923) also attested to this concept of the histology of the glycogen body. Terni (1924) detected glycogen in the tissue and contended that this material was retained within the cells.

It is understandable why such differences of opinion arose in view of the fact that it is quite difficult to recognize individual cells in the tissue of the glycogen body once that structure has taken a definite form in the embryo. The cells are fitted to one another in such a way that their individual outlines are obscured. Moreover, unless the glycogen body is fixed and stained for glycogen, the tissue appears to consist of a series of fibrils which are derived from the small amount of cytoplasm around the nucleus of each cell. In the present study, the use of protargol enhanced the reticular appearance of the tissue. It would seem that Imhof was misled into believing that the glycogen body consisted of star-shaped cells, because he stained only the small amount of cytoplasm which surrounds the nucleus of each cell. It seems that he also mistook the deposition of silver stain between adjacent cells to be the cytoplasmic processes or extensions of the cells.

Duval (1877), and Terni (1924) maintained that the cells of the glycogen body were oval or spherical in form but appeared as polyhedrons because they were pressed tightly against each other. This suggestion is supported by the results of the present study in which it has been found that the majority of cells appeared as polyhedrons in the embryo, but are oval or spherical in form in chorio-allantoic grafts and in cell culture. These results are best explained by assuming that growth took place in relatively unconfined environments in which there was little or no pressure exerted upon the cells as compared to the restricted area of the lumbo-sacral sinus of the embryo. Terni's contention that the cells of the glycogen body increase in size and proliferate by mitosis even though they are filled with glycogen is supported by the results of this study. The increase in the size of the glycogen body cell which is noted during normal development and in chorio-allantoic grafts, clearly indicates that the increase in cell size is a definite characteristic of differentiation for the glycogen body. It seems apparent from the results of the present study that growth and

differentiation of the glycogen body involves an increase in the number of cells, an increase in the size of the cell and the accumulation of glycogen.

Differentiation and Glycogen Accumulation

Doyle and Watterson (1949) made quantitative determinations of the glycogen in the glycogen body and estimated that the glycogen body represents about 5% of the glycogen in the chick embryo at 15 days of incubation. These authors noted that the most rapid period of glycogen accumulation was between 10-14 days. Jenkins (1955) suggests that some relationship exists between the fall in liver glycogen and the accumulation of glycogen by the glycogen body at 12 days of incubation. Watterson *et al.* (1958) reported that the glycogen content of the glycogen body was less in hypophysectomized chick embryos when compared to the unoperated controls. These authors found that there was no difference between the experimentals and controls until after 12 days of incubation. They concluded that the accumulation of glycogen by the glycogen body after 12 days of incubation is directly or indirectly dependent upon the production of pituitary hormones.

While it is probable that the overall amounts of glycogen which can be stored by the glycogen body may be influenced by hormonal or other physiological relationships which may arise during development, the results of the present investigation suggest that such interactions are not primarily responsible for the initiation and differentiation of glycogen-body cells and formation of glycogen by the cells of the glycogen body. This idea is supported by the finding of glycogen body cells in chorio-allantoic grafts and in cell culture. The results obtained from chorio-allantoic grafting indicate that the presumptive cells of the glycogen body are present in the presumptive sacral region of the nerve cord as early as 33-48 hours of incubation. Since glycogen did not appear in these cells until 7-8 days of incubation, there may be some specific events at the 7-8 day period which began to initiate and govern the growth of the glycogen body in the embryo. The fact that the glycogen body is able to differentiate on the chorio-allantoic membrane eliminates the possibility that growth and differentiation of that tissue is initiated by influences which could be conveyed to the lumbosacral region along nerve tracts from higher centers in the nervous system. Secondly, the size of the glycogen body cell (shown to be an indication of the degree of differentiation) found in chorio-allantoic

grafts is always close to the cell size of the control glycogen body. If the presumptive cells of the glycogen body accumulated glycogen during the 10 days they were grafted to the chorio-allantoic membrane, one would expect their size to increase to that found in a glycogen body that had been accumulating glycogen for 10 days, that is, in a glycogen body of 17-18 days of incubation. Such was the case in the 7-8 day donors where the size of the glycogen body cell in chorio-allantoic grafts indicated a degree of differentiation which could be found in a control glycogen body of about 16 days of incubation. In 2- and 3-day donors, however, the size of the cell was indicative of a glycogen body of 10 and 12 days and not that of 17 days. These results strongly suggest that the stem cells of the glycogen body did not differentiate and accumulate glycogen until they had reached 7-8 days of age in spite of the fact that they were transplanted to the chorio-allantois of an 8-9 day embryo and were exposed to the environment of the host from that time on.

The results obtained with cell-culture experiments in the present study indicate that glycogen body cells from 12- and 14-day embryos as well as the stem cells from 6- and 8-day embryos were able to proliferate and accumulate glycogen *in vitro*. These results suggest that the cells of the glycogen body are not dependent upon the immediate environment of the embryo for growth and differentiation. Kwiak (1956) cultured explants of glycogen body from 10-17 day chick embryos using embryo extract and chicken serum. He found that growth was greatest in cultures containing 12-day glycogen body. This agrees with the results of the present study in which it was found that cell cultures of lumbo-sacral nerve cord from 12-day and 14-day embryos showed the greatest increase in glycogen body cells after culture. In the case of the present study, however, cell counts were made in order to learn whether there was an increase in the number of cells in a culture rather than to obtain an index of the growth of the glycogen body in embryos of different ages.

The results of the present study offer little information to suggest a function for the glycogen body. Attempts by other workers to prove that the glycogen body is a metabolic source of energy for the post-hatching period of the bird have met with little success (Terni 1924, Doyle and Watterson 1949, Watterson *et al.* 1958), but one cannot help agreeing with Watterson (1949) that future research will probably show that the glycogen body plays an important role in avian development.

Summary

1. Differentiation of the glycogen body of the chick embryo was studied under normal developmental, chorio-allantoic and *in vitro* conditions using histological and cytochemical techniques.

2. The earliest indication of the formation of the glycogen body in the chick embryo is noted at 7-8 days of incubation when glycogen-storing cells appear in the medial and lateral parts of the roof plate at the lumbo-sacral level of the nerve cord.

3. The tissue of the glycogen body consists of glycogen-storing cells which are oval or spherical in form. Glycogen is found in these cells during incubation and on the first day of hatching.

4. Growth and differentiation of the glycogen body involves an increase in the number of cells, an increase in the size of the cell and the accumulation of glycogen.

5. Growth and differentiation of the glycogen body occurs when presumptive sacral tissue and nerve cord is isolated from 7-8 day, 3-4 day and 33-48 hour embryos and transplanted to the chorio-allantoic membranes of 8-9 day hosts.

6. Glycogen body cells and nerve cord isolated from the lumbo-sacral region of 12-day and 14-day embryos, as well as the presumptive cells and nerve cord from 6-day and 8-day embryos proliferate and accumulate glycogen when cultured *in vitro*.

7. It is suggested that the glycogen body is not dependent for growth and differentiation upon influences originating at higher levels in the nervous system and conveyed within the nerve cord to the lumbo-sacral region, but arises in the chick embryo primarily by an inherent capacity of the cells to synthesize glycogen.

Acknowledgment

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References

1. Bodian, D. 1937. The staining of paraffin sections of nervous tissue with activated protargol. *Anat. Rec.*, **69**, 153-162.
2. Doyle, W. L., & Watterson, R. L. 1949. The accumulation of glycogen in the glycogen body of the nerve cord of the developing chick. *J. Morph.*, **85**, 391-404.
3. Duval, M. 1877. Recherches sur le sinus rhomboidal des oiseaux. *J. de L'anat. et la physiol.*, **13**, 1-38.
4. Eagle, H. 1955. Nutritional needs of mammalian cells in tissue culture. *Science*, **122**, 501-504.
5. Gelfant, S., Meyer, R. K., & Ris, H. 1955. Uterine growth following stimulation by estrogen and inhibition by aminopterin and nitrogen mustard. *J. Exp. Zool.*, **128**, 219-257.
6. Gomori, G. 1952. Microscopic Histochemistry: Principles and Practice. The University of Chicago Press, Chicago.
7. Hamburger, V., & Hamilton, H. L. 1951. A series of normal stages in the development of the chick embryo. *J. Morph.*, **88**, 49-92.
8. Hansen-Prus, O. C. 1923. Meninges of birds, with a consideration of the sinus rhomboidalis. *J. Comp. Neur.*, **36**, 193-217.
9. Hoadley, L. 1924. The independent differentiation of isolated primordia in chorio-allantoic grafts. *Biol. Bull.*, **46**, 281-315.
10. Imhof, G. 1905. Anatomie und Entwicklungsgeschichte des Lumbalmarkes bei den Vögeln. Arch. für mikroskopische Anatomie und Entwicklungsgeschichte, **65**, 498-610.
11. Jenkins, F. A. 1955. Liver glycogen storage in the chick embryo and its relation to the glycogen body. *Wasmann J. Biol.*, **13**, 9-35.
12. Kwiak, J. 1956. Cialo glikogenowe kurcsecia w hodowli tkanek. (The glycogen body of the chick in tissue culture.) *Folia Morph.*, **7**, 125-132.
13. Leydig, F. 1854. Kleinere Mittheilungen zur thierischen geweblehre. *Arch. fur Anatomie und wissenschaftliche Medicin*, 296-348.
14. McManus, J. F. A. 1948. Histological and histochemical uses of periodic acid. *Stain Techn.*, **23**, 99-108.
15. Terni, T. 1924. Ricerche sulla cosidetta sostanza gelatinosa (corpo glicogenica) del midolla lumbo-sacrale delgi uccelli. *Arch. Ital. di Anat. e di Embriol.*, **21**, 55-86.
16. Ungewitter, L. 1943. A controllabel silver stain for nerve fibers and nerve endings. *Stain Techn.*, **18**, 183-186.
17. Watterson, R. L. 1949. Development of the glycogen body of the chick spinal cord. I. Normal morphogenesis, vasculogenesis and anatomical relationships. *J. Morph.*, **85**, 337-390.
18. ———, Veneziano, P., & Brown, D. A. 1958. Development of the glyocen body of the chick spinal cord. V. Effects of hypophysectomy on its glycogen content. *Physiol. Zool.*, **31**, 49-59.
19. Wright, B. S., & Sagik, B. P. 1958. Plaque formation in monolayers of chicken embryo kidney cells. *Virology*, **5**, 573-574.

Growth, 1959, **23**, 251-271.

A GRADIENT OF PROTEIN SYNTHESIS IN PLANARIA AND REVERSAL OF AXIAL POLARITY OF REGENERATES

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INTRODUCTION

SEQUENTIAL differentiation along polar axes is a striking feature of early embryonic development as well as in the regeneration of such forms as hydroids and flatworms. The axial gradient theory developed by Child (1907, 1941) postulated that quantitative axial gradients of physiological activity are causal factors underlying axial patterns of differentiation. Such a speculation seems important in view of the experimentally demonstrated totipotency of the various parts of early stages of developing embryos and regenerating hydroids or flatworms. However, it is difficult to conceive the role that gradients play in the complex morphogenesis of later development. The author has been led to the hypothesis that embryonic induction is any one of a number of stimuli which can evoke protein synthesis in the reacting tissue (Flickinger, 1958b) and has implicated the regional and temporal sequence of conversion of yolk protein to cytoplasmic protein as a causal mechanism in the differentiation of cells of the amphibian embryo (Flickinger, 1957, 1958a; Rounds and Flickinger, 1958).

The well-known ability of a cut section of a planarian to regenerate a head at the anterior end and a tail at the posterior end is a classic example of differentiation in a polar system. Any level of a decapitated worm can form a head, but the head invariably forms at the anterior end of a cut section of worm if the piece is long enough. Child's gradient theory attempts to explain this by proposing that the higher level of physiological activity at the anterior end causes the head to form there. Brønsted (1955), DuBois (1949) and Lender (1956) have recently published interesting contributions to the study of flatworm regeneration.

[1] This research was supported by a grant from the National Science Foundation.

Flickinger and Blount (1957) recorded bioelectric potentials, and also made polarographic respiration measurements with recessed platinum electrodes, but were unable to detect bioelectrical or respiratory gradients. Hyman and Bellamy (1922) recorded bioelectrical and respiratory gradients in planaria and Hyman (1932) demonstrated a respiratory gradient in Nereis, but Lovtrup (1953) could not demonstrate a respiration gradient in flatworms using the Cartesian diver technique. Flickinger and Blount found that when an E.M.F. was applied along the anterior-posterior axis of a planarian, and respiration measurements were made at the same time, that the higher imposed potentials induced a respiratory gradient in the flatworm. The imposed potential was similar in magnitude to that used by Marsh and Beams (1952) in reversing the axial polarity of regenerating planaria.

Recently Kanatani (1958) has found that treatment of whole worms with deacetylmethylcolchicine for two days prior to cutting produced a large percentage of bipolar heads from transverse sections. This colchicine derivative was much more effective than the parent compound which has been shown to inhibit division of free parenchymal amoebocytes in the planarian (McWhinnie, 1957).

If the hypothesis that differentiation is causally allied with protein synthesis is true, then it seemed important to determine if the rate of protein synthesis in planaria varied along the anterior-posterior axis of the worms. Verification of this idea would provide some support for Child's theory. Chloramphenicol (chloromycetin), an inhibitor of protein synthesis, and deacetylmethylcolchicine (colcemide-Ciba)[2] were applied to the anterior ends of regenerating worms in an attempt to modify any gradient in rate of protein synthesis that might exist and then the effect upon the biological polarity of the regenerating worms was determined.

Materials and Methods

The flatworms used were *Dugesia tigrina* and they were kept in a refrigerator at 10°C. without being fed. The worms were maintained in boiled, filtered, Cl_2-free tap water. The incubation of worms with isotope ($C^{14}O_2$ or glycine-1-C^{14}) was carried out in the following

[2] I am pleased to acknowledge a complimentary sample of colcemide from the Research Department of Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

manner. In the case of glycine C-14, 100-250 worms were placed in small stender dishes containing 2 cc. of tap water with 3 mg. (55 μc) of glycine C^{14} added. The duration of these incubations was 2 hours at 23°C.; the $C^{14}O_2$ incubations were for an hour. For the $C^{14}O_2$ incubations 100-250 worms were placed in 2 cc. of tap water in the bottom of a 15 cc. Warburg flask, 0.2 mg. (15 μc) of $BaC^{14}O_3$ was added to the side arm and a small amount of lactic acid was added to the side arm to dissolve the $BaC^{14}O_3$. The flask was closed immediately upon addition of the lactic acid. In both types of experiment the vessels were rotated gently upon an automatic platform shaker during the incubation. At the termination of incubation the worms were fixed in cold 5% trichloracetic acid and then cut with a steel needle under the dissection microscope into 3, 4, 5 or 6 pieces of equal size.

The various regional fractions were then homogenized in cold 10% TCA and, after removal of the acid soluble fraction by centrifugation and washing, the nucleic acids were removed by hot 5% TCA (90° for 10 minutes), and hot 3:1 alcohol-ether, 1:1 alcohol-ether, and ether were used to remove the lipids and any contaminating TCA. Several washings were employed in each type of extraction. The acid insoluble fractions minus nucleic acids and lipids were considered to be primarily protein. In some cases the nucleic acids and lipids were not extracted and total acid insoluble fractions were used. The samples were then dried in an Abderhalden drier for several hours at the temperature of boiling chloroform, then converted to CO_2 by combustion, and collected as $BaCO_3$ in a 1% $BaCl_2$-saturated $BaOH_2$ solution. Thirty or forty milligrams of dried $BaCO_3$ were plated out on planchets by centrifuging $BaCO_3$ down from an acetone suspension. These samples were counted on a windowless flow counter.

In the experiments in which the anterior ends of cut pieces of planaria were exposed to various concentrations of chloroamphenicol or colcemide, small lateral incisions were made on the sides of the pieces in the anterior region at the time of cutting the transverse sections of the worms. These wounds identified the anterior ends of the pieces. The worms were allowed to heal 3-4 hours at room temperature (23°C.) and overnight at 6°C. before being used in the experiments. Usually mid-pieces, which were about ⅓ the length of the whole worm and included the pharynx, were used in these experiments, but pre- and postpharyngeal pieces were also used in these experiments, and pieces of various length were also used. When pieces of worms

that were ⅔ the length of the worm (as long as the prepharyngeal and mid-regions) were used, the percent reversal was very low and these are not recorded. In some cases heads were left on the prepharyngeal pieces and the head ends placed in the test solutions in order to test for any inhibiting action of the head in preventing the differentiation of a new head at the posterior ends of the pieces. The pieces were embedded in thin slabs of 2% agar, while the agar was solidifying, and the anterior ends were placed down into the solutions of chloramphenicol or colcemide as shown in Fig. 1.

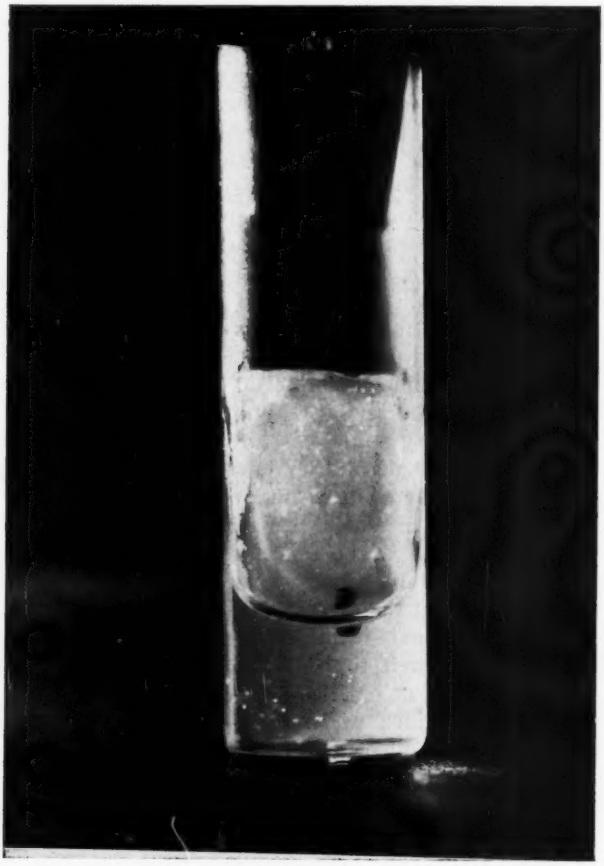

FIGURE 1
An agar-slab experiment in which the anterior end of a regenerating fragment of a flatworm was exposed to chloramphenicol.

In an attempt to discern a quantitative distribution of proteins in intact worms, rabbit antisera were built up against homogenates of isolated anterior and posterior halves of numerous sectioned flatworms. These antisera were used in precipitin reactions and also for Ouchterlony agar-plate diffusion tests (Björklund, 1952). Over 1,000 worms were cut and 4 cc. of homogenate from each fraction was injected intraperitoneally in one, one and two cc. amounts on alternate days. The rabbits were bled seven days after the last injection. Both the antisera to the anterior and the posterior fractions reacted with the centrifugal supernate of the homologous antigen at a level of .027 mg. protein/cc. as determined by Nesslerization experiments. The anterior fraction antiserum showed a positive precipitin reaction after six hours, while the posterior fraction antiserum gave a positive precipitin reaction after seven hours.

Results

Isotopic Experiments

The results of incubating whole planaria in $C^{14}O_2$ and glycine C-14 and determining the specific activity of the protein fractions from various regions of the worms are illustrated in Figs. 2 and 3. In both the $C^{14}O_2$ and glycine C-14 experiments there was some variation in the amount of isotope administered, the number of worms used and the duration of incubation. Since the main point of interest in these investigations was to ascertain if a gradient exists, the fact that it was impossible to compare absolute specific activities between different experiments was not important. In Fig. 2 a family of curves illustrating regional $C^{14}O_2$ incorporation into the protein fractions are represented and the glycine C-14 results are given in Fig. 3. Each curve represents one experiment in which regions of 100-250 worms were combusted and counted. It is to be noted that the worms were cut differently in the various experiments. They were cut into 3, 4, 5, or 6 equal sized pieces and hence the number of determinations for whole worms varies between different experiments. It is clear from examination of Figs. 2 and 3 that an axial gradient of incorporation of both $C^{14}O_2$ and glycine C-14 into protein occurs, with the head possessing the highest activity. In the case of glycine the tail regions show an increased activity which is greater than that of the mid-section, but a good deal less than that of the head. These experiments do not indicate which cell types are most active in the uptake of carbon-14. The

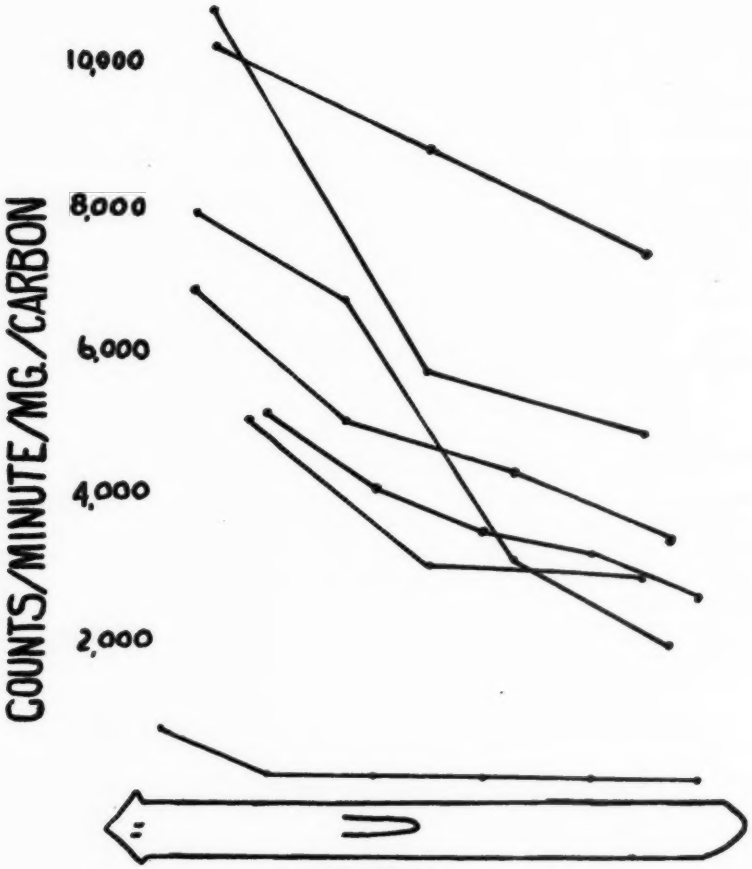

FIGURE 2

Specific activity of the protein fractions from various regions of the worms after incubation with $C^{14}O_2$. The number of points on the curves represent the number of regional fractions and the points lie at the middle of any regional fraction as projected up from the drawing of the flatworm beneath.

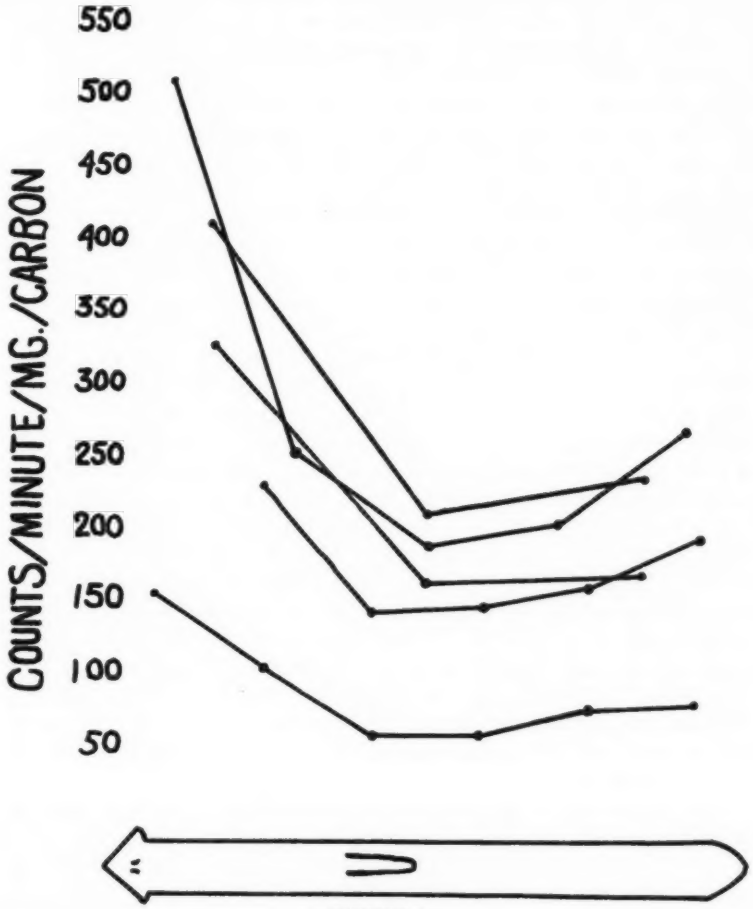

FIGURE 3

Specific activity of the protein fractions from various regions of the worms after incubation with glycine C-14. The points are represented in the same manner as in Fig. 2.

incorporation of carbon-14 into proteins is thought to be a measure of protein synthesis since the incorporation is inhibited by chloramphenicol, a known inhibitor of protein synthesis. Due to lack of knowledge about the existence of net synthesis or tissue turnover, in intact flatworms, the incorporation rates might best be considered a reflection of some aspect of the protein synthesizing mechanism(s). For ease of presentation incorporation is considered as synthesis.

In four series of glycine C-14 experiments the living worms were cut in the manner shown by the solid lines in column 1 in Fig. 4A and B so as to provide either one or two pieces from each worm, each piece regenerating a head and a tail. Regeneration was allowed to proceed for varying lengths of time and then regenerating pieces were incubated with glycine C-14, fixed, cut into regional fractions as shown in the second column in Fig. 4A and B, extracted, and the specific activities of the protein fractions were estimated. In one experiment (4C) tails were cut off of a number of worms, regeneration was allowed to proceed for 1½ days and the isotope activity of the posterior ⅓ of the worms (including the regenerating tails) was then compared to that of the anterior third (the middle fraction was lost in this case).

These results are outlined in Fig. 4A, B and C, with the specific activities listed directly by the regional fraction in question. For the two experiments outlined in Fig. 4 A the cut pieces in experiment 1 were allowed to heal two hours at 23°C. and twenty hours at 10°C. before being fixed and cut. The pieces of experiment 2 (4A) were fixed and cut immediately after the living worms were sectioned (it took about 2 hours to cut the living worms). For the experiments illustrated in 4B the cut worms were allowed to heal for 2 days in experiment 1 and 20 hours in experiment 2 before being fixed in cold 5% TCA and cut.

The regenerating pieces tend to show about the same regional pattern of incorporation of glycine C-14 as similar regions of intact worms. Some increase in glycine incorporation can be noted in the regenerating regions. It is to be noted in 4B (experiment 2) that the posterior ends of the cut sections of the worms have a higher incorporation of isotopic glycine into proteins than do the anterior ends, although the anterior ends will form the heads and the posterior ends will form the tails. These are the same regional glycine C-14 activities one finds in the intact worms, with the tail having a higher activity than the regions just ahead of it. This does not necessarily imply that the rate

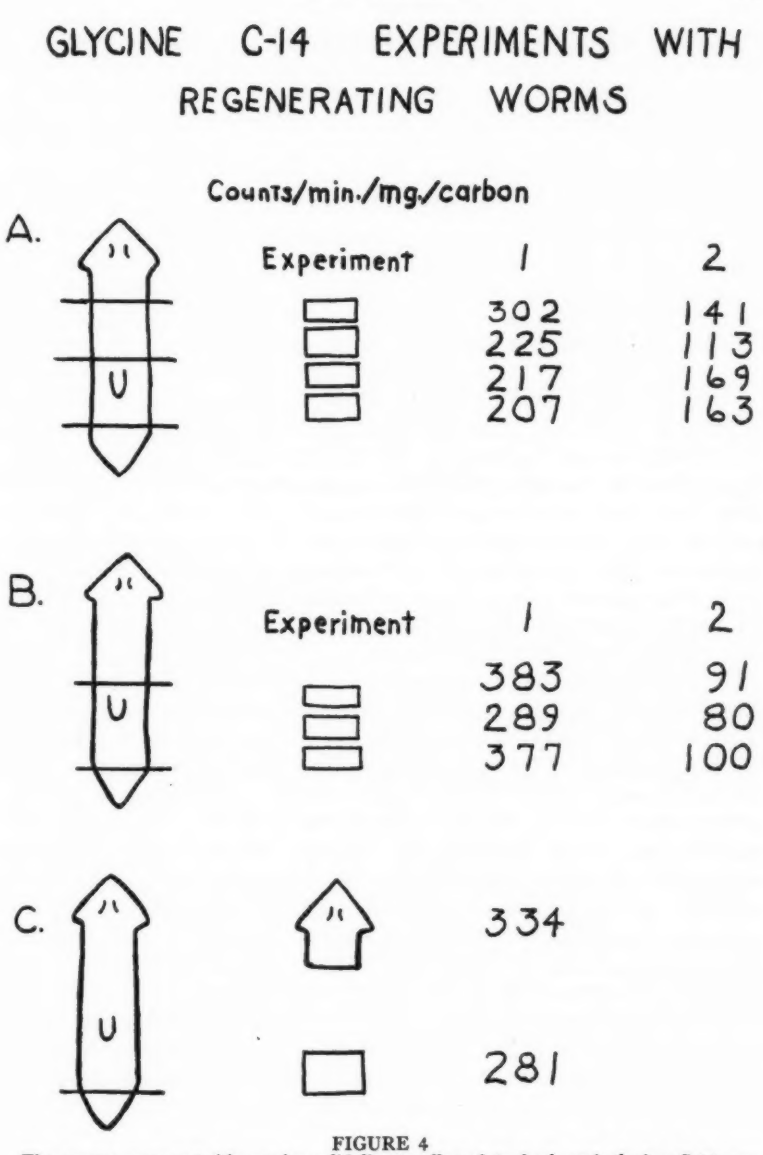

FIGURE 4

The worms were cut (shown by solid lines), allowed to heal, and glycine C-14 was administered at various periods during regeneration. The isotopic incorporation into the protein fractions from the regions of the regenerating pieces (seen in column 2) is represented as counts/min./mg./carbon.

of protein synthesis at the posterior ends of these pieces of worms is higher since the incorporation of $C^{14}O_2$ does not show an elevated activity in the tail. If one speaks about a gradient of isotopic carbon-14 incorporation into proteins, it becomes necessary to stipulate the isotope used. To gain a complete picture of protein synthesis it would be necessary to utilize all the precursor molecules for the various proteins. Certainly CO_2 and glycine are utilized in a different way by the worms. In this case the results do not necessarily negate the speculation that the head forms at the site of more rapid protein sythesis in a sectioned planarian. Evidence of a more critical nature on this question was subsequently obtained from the experiments with chloramphenicol and colcemide.

Serological Experiments

In order to test the idea that there might be a regional distribution of different specific proteins in the worms, which might account for retention of the original polarity in regenerating worms, the following serological experiments were performed with antisera to isolated anterior and posterior halves of flatworms. At test antigen concentrations of 0.113 mg. protein/cc. the antiserum to the anterior halves of the worms showed a positive reaction with the anterior fraction test antigen after ten minutes, but did not react with the posterior fraction test antigen until 1½ hours. At test antigen concentrations of 0.068 mg. protein/cc. the antiserum to the posterior fraction showed a positive precipitin reaction with the anterior fraction test antigen after two hours but had not shown a positive reaction with the homologous antigen (posterior fraction) after five hours. Although the antiserum to the anterior halves of the worms reacts more readily with the anterior than the posterior test antigen, the antiserum to the posterior fraction also reacts more readily with the anterior test antigen at equal protein concentrations of both test antigens. These results were clarified by Ouchterlony agar-plate diffusion experiments. If the antiserum to the anterior halves of worms is allowed to react with the anterior and posterior test antigens two broad, well defined lines of antigen-antibody precipitate appear and these lines meet and fuse to show the reaction of identity. There is another fainter line which apparently also shows the reaction of identity. The serological results seem to demonstrate the similarity of the main antigenic components of the anterior and posterior fractions.

Effect of Chloramphenicol and Deacetylmethylcolchicine (Colcemide) on Polarity of Regenerating Worms

Before utilizing chloramphenicol and colcemide, several other types of experiments were performed. First of all the rate of regeneration of eye spots for anterior pieces cut just behind the head was compared with pieces of worms in which the anterior cut was made just anterior to the pharynx. The lengths of these pieces were similar. It was observed that eye spot regeneration was slightly faster in the anterior pieces. Faint eyespots usually appeared one day earlier in the anterior than in the posterior pieces. In an effort to accelerate regeneration various concentrations of a yeast extract, proteose-peptone, dextrose solution and a nutrient solution containing various amino acids, dipeptides, vitamins, nucleotides, sugars and sodium adenosine triphosphate were used, but they did not affect the rate of regeneration as estimated by the time of appearance of the eyespots. Bubbling CO_2 into .01 M trishydroxymethylaminomethane buffered tap water was also without effect upon rate of regeneration. Solutions containing nucleic acid antagonists (benzimidizole, 2-amino-4 methyl pyrimidine, 2,6-diaminopurine sulfate, 1 mg. each/cc. tap water) and amino acid antagonists (ethionine, methionine sulfoxide, β phenyl lactic acid, allylglycine, 2 mg. each/cc. tap water) were both effective in retarding the rate of regeneration. However, neither of these solutions was as effective as chloramphenicol or colcemide so that the latter two compounds were used in the agar-slab technique (Fig. 1) to expose the anterior ends of the worms to the test solution. Chloramphenicol (0.75 mg./cc. of tap water) had a definite retarding effect upon the regeneration, as judged by the time of appearance of the eyespots of cut sections of worms placed in the solution. Eyespots in control animals appeared by six days but did not appear until the ninth or tenth day in the chloramphenicol treated worms. After fourteen days, when the controls had large normal eyes, the eyespots of the chloramphenicol worms were quite small.

A higher concentration of chloramphenicol (1 mg./cc. of tap water) was used in the agar-slab experiments. The concentration of colcemide was 0.02 mg./cc. (M/20,000) in tap water. The cut sections were allowed to heal and were then embedded in 2% agar and the agar-slabs containing the worms were cut out and placed so that the anterior ends were in the solution and the posterior ends were not

(Fig. 1.). The slabs were left in chloramphenicol for three or four days and in colcemide for one or two days. After this treatment the worms were removed from the agar and were placed in tap water and examined every day. The healed lateral incision at the anterior end identified it as anterior and also served to show if any of the pieces had changed position while in the agar. The few that had reversed position were discarded. In the colcemide experiments whole worms were first exposed to colcemide (0.02 mg./cc.) for one day, then the worms were cut and allowed to heal in tap water and then embedded in agar. The results of the chloramphenicol and colcemide experiments are expressed in Table 1, and a photograph of a bipolar worm from

TABLE 1
AGAR-SLAB EXPERIMENTS

	No. of Cut Pieces	Normal Polarity	Inhibition of Regeneration	Bipolar Development	Reversal of Polarity
Chloramphenicol (1 mg./1 cc.)					
Postcephalic pieces	91	62	1	8	20
Cephalic pieces (with heads)	46	46	0	0	0
Colcemide (0.02 mg./cc.)					
Postcephalic pieces	103	74	2	20	7
Cephalic pieces (with heads)	30	30	0	0	0

colcemide treatment is shown in Fig. 5. Control experiments, in which anterior ends of cut worms in agar were exposed to seitz filtered tap water (containing penicillin and streptomycin to prevent contamination), did not show reversals of polarity. These data clearly show that chloramphenicol can reverse the polarity of regenerating planaria in a significant percentage of the cases, while colcemide induces a bipolar development in a large number of cases. It is likely that a higher incidence of reversal was not obtained because of the difficulties of the agar-slab method. It is not certain that a true unipolar exposure of the cut piece to the solution occurs in all the agar-slab cases. The use of a neural red dye solution in an agar-slab experiment indicated that some dye did diffuse to the unexposed end of the agar, but the dye set up a gradient diffusion pattern along the long axis of the worm. It is to be noted that the presence of heads on the pre-pharyngeal pieces

FIGURE 5
A bipolar worm produced by M/20,000 colcemide (0.02 mg./cc.) in an agar-slab experiment.

does not permit reversal or bipolarity. It is known that the presence of a head seems somehow to inhibit the formation of another head at the posterior cut surface of a very short piece of worm; such a piece would ordinarily become bipolar if the old head were not present (Child, 1941).

The explanation of these reversals of polarity of the regenerating planaria by exposure of the anterior ends to chloramphenicol and production of bipolars by colcemide is believed due to retardation of the rate of protein synthesis at the anterior ends of the sectioned worms. Chloramphenicol is known to inhibit or retard protein synthesis and colcemide would be expected to retard the rate of protein synthesis by virtue of its property of inhibiting cell division. The fact that very few cases of reversed polarity, but many cases of bipolar head development, were obtained with colcemide in the agar-slab experiments may be due to the prior exposure of whole worms to colcemide.

To investigate further the possible role of the head in inhibiting the formation of another head at the sectioned posterior end of a planarian, Kanatani's (1958) experiments with deacetylmethylcolchicine were repeated with several modifications. Intact worms were exposed to colcemide (0.02 mg./cc.) for one day and then cut into thirds consisting of postpharyngeal pieces with the tails cut off, pharyngeal pieces and prepharyngeal pieces with the heads cut off. In a number of cases the heads were left on the prepharyngeal pieces. These worms were allowed to heal and regenerate in tap water. Kanatani's (1958) results which showed that prior treatment of intact worms with colcemide can elicit bipolar head formation in a large percentage of the sectioned worms were confirmed. The incidence of bipolar heads in the present work was thirty percent. Examination of prepharyngeal pieces with the heads left on reveals that even in colcemide treated worms the presence of the head interferes with head development at the cut posterior end as revealed by the lack of bipolar development in these cases.

In order to ascertain if chloramphenicol and colcemide are affecting the biological polarity of regenerating flatworms by means of abolishing or reversing the natural gradient of rate of protein synthesis (see Figs. 2 and 3), the following experiments were carried out. Approximately 100-150 worms were placed in chloramphenicol (.75 mg./cc.) for a day and then they were incubated in a fresh solution of chlor-

amphenicol for one hour while also being exposed to $C^{14}O_2$ in a Warburg flask. The worms were fixed in cold TCA and cut into four pieces and the protein fractions were obtained, converted to $BaCO_3$ by combustion and counted. Examination of Table 2 shows that the gradient of protein synthesis was not greatly altered in such an experiment. However, if the worms were in a 1 mg./cc. concentration of chloramphenicol for two days, and then incubated with $C^{14}O_2$ while in that concentration of chloramphenicol, the incorporation of $C^{14}O_2$ into various regional fractions was drastically inhibited (Table 2). This impairment of $C^{14}O_2$ incorporation into protein by chloramphenicol suggests that the isotope incorporation may be in part a reflection of protein synthesis. Although the gradient pattern was not abolished by chloramphenicol, its steepness was altered. Also the activity of the regional fraction just in front of the tail was essentially similar to that of the tail. Several experiments were performed in which worms were exposed to colcemide (0.02 mg./cc.) for 1 day and then incubated in colcemide-$C^{14}O_2$ for one hour. The acid fixed worms were cut into four pieces and the specific activities of the total acid-insoluble fractions from the four parts of the worms were determined as shown in Table 2. It seems that colcemide can abolish

TABLE 2
$C^{14}O_2$ EXPERIMENTS WITH CHLORAMPHENICOL AND COLCEMIDE TREATED WORMS

	Counts/minute/mg./carbon in the four regions of treated worms			
Chloramphenicol	1 (Head)	2	3	4 (Tail)
0.75 mg./cc. for 1 day	2,058	1,013	813	664
1.0 mg./cc. for 2 days	249	221	187	186
Colcemide	554	561	455	571
0.02 mg./cc. for 1 day	796	631	531	714
	594	364	408	407

the axial gradient of protein synthesis in the postcephalic regions. In the colcemide experiments the gradient has been altered as witnessed by the higher specific activity of the tail fractions as opposed to the two middle regions. In one experiment the gradient was completely abolished since the specific activity of the head fraction was no greater than in any other part.

The fact that colcemide treatment does not ordinarily reduce the rate of protein synthesis of the head region to the level of the postcephalic regions may account for the inability of prepharyngeal pieces

with the heads present to form heads at their posterior cut ends under conditions of chloramphenicol or colcemide treatment. It is to be noted that the ratio of the rate of protein synthesis between the head and tail regions is reduced from the average normal head/tail ratio of 2.8/1 to about 1.1/1. Although the effect of colcemide in altering or abolishing the gradient in rate of incorporation of $C^{14}O_2$ into proteins, which is presumably a gradient of rate of protein synthesis, does not prove that this particular action of the compound is instrumental in eliciting bipolar cephalic development in regenerating worms, the correlation is indeed suggestive. Also the fact that a known inhibitor of protein synthesis, chloramphenicol, can evoke reversal of polarity in the agar-slab experiments provides an argument for the contention that the site of head formation in a cut section of a flatworm takes place at the end with a higher rate of protein synthesis. Head formation does seem to be independent of the absolute rate of protein synthesis over a wide range.

Discussion

The isotopic evidence presented in this paper is consistent with the concept of an axial gradient of incorporation of $C^{14}O_2$ into the protein fraction of flatworms. In the case of glycine C-14 the gradient is modified by an increased specific activity in the tail region which is greater than the pharyngeal, but less than the prepharyngeal region. It is presumed that these gradients of incorporation may be in part gradients of rate of actual protein synthesis, since they can be altered by chloramphenicol, a known inhibitor of protein synthesis. Isotopic experiments upon worms that have been cut, and are regenerating, indicate that the glycine incorporation gradient prevails immediately after cutting, but it is modified during the first few days of regeneration by stimulation of incorporation of glycine C-14 in the areas of regeneration.

By using antisera to the anterior and the posterior halves of flatworms the question of gradients of head and tail forming substances was investigated by means of precipitin reactions and Ouchterlony plate serology. Certainly there are proteins in the differentiated head structures (e.g., eyes) that are not present in comparable quantity in the body of the worm; however, the evidence indicated that the main serological components were not present in a gradient pattern along the axis of the worms. The fact that a short cephalic piece, obtained

by a posterior cut in back of the head, regenerates a tail does not fit the concept of a gradient of head forming substances since one would expect more head substances than tail substances at this anterior level.

Since a head or a tail can form at any given level of a worm and this level, just after cutting, has the same rate of protein synthesis and presumably the same amount of substances (either of a stimulating or inhibiting nature for head or tail formation), this places the emphasis upon competitive reactions along the anterior-posterior axis of the worms.

In an article dealing with the problem of apical dominance in plants Gregory and Veale (1957) consider the mechanism by which the apical growing bud inhibits the growth of lateral buds. They compare the evidence suggesting that specific substances (auxins) inhibit lateral growth with the notion that lateral inhibition is due to a lack of nutrient materials which are present in a limited supply and for which the apical bud competes most successfully. The fact that apical dominance can be maintained by placing auxin on a decapitated stem seems to be explained by auxin impeding the differentiation of vascular connections to the axillary buds and thus limiting their nutrient supply. Gregory and Veale present the results of experiments with flax seedlings that show the degree of apical dominance to be determined by the nitrogen and carbohydrate supply of the plant. With a high nitrogen supply apical dominance is almost absent while it is complete under conditions of nitrogen starvation. Hence competition for a limited nutrient supply appears to account for apical dominance and inhibition of lateral buds. An analogy may be drawn between this situation in plants and regeneration in flatworms. In planaria an axial gradient of protein synthesis has now been demonstrated and removal of the site of highest rate of protein synthesis (the head—see Figs. 2 and 3) allows head production at a posterior level, just as decapitation of the apical bud allows growth of some of the lateral buds, presumably due to removal of the site of most avid competition for nutrients. Reversal of polarity of regenerating planaria by chloramphenicol, and formation of bipolars by colcemide, also fits the hypothesis that competition for nutrient for protein synthesis is a decisive factor in establishing polarity in regenerating flatworms.

$C^{14}O_2$ experiments with chloramphenicol and colcemide treated worms indicate that these compounds abolish or alter the axial

gradient in protein synthesis at postcephalic levels of the worms (Table 3). It is presumed that chloramphenicol treatment of the anterior ends of cut planaria is reversing metabolic polarity since the posterior ends of these pieces are not exposed to the inhibitors of protein synthesis (Fig. 1). It seems logical to attempt to explain the results by assuming that a reversal of the normal axial gradient of protein synthesis is eliciting a reversal of biological polarity in the regenerating worms. Miller (1939) has induced reversal of polarity in regenerating hydroid stems by increased temperature at the basal ends of the stems. In this case it is assumed that the higher temperature confers a higher rate of metabolism to the basal end thus favoring hydranth formation at this site.

In planarian regeneration if the head does form at the end with the higher rate of protein synthesis, this is somewhat similar to the case of the frog gastrula in which the "organizer" forms at the primary site of yolk utilization according to Flickinger (1957). Since cytoplasmic protein is in large part synthesized at the expense of the yolk, this implies that this is the site of optimal conditions for cytoplasmic protein synthesis.

In early embryonic development there is selective sequential differentiation, with the various tissues and organs appearing in a definite temporal fashion. One possibility is that certain tissue and organ specific protein synthesizing mechanisms are the most efficient competitors for materials necessary for protein synthesis and therefore their specific protein populations (whose properties are in part perhaps responsible for differentiation) are synthesized prior to other specific proteins and accordingly tissues and organs appear in a sequential fashion.

The pattern of morphogenesis and differentiation, according to the speculations presented here, might possibly be ascribed to an interaction between two variables. One of these would be the quantitative variation of conditions necessary for protein synthesis, taking into account all of the necessary factors involved (Flickinger, 1958b). Then there is a subsequent competition between protein synthesizing mechanisms, but the competition is pre-judged since the second variable is a sequential hierarchy, or order, of success in the competition between tissue and organ specific protein-synthesizing mechanisms whose products account in part for differentiation. To utilize classical terminology one might apply certain aspects of the term "fields" to the

first variable and certain aspects of "competence" to the second variable. In this hypothesis the tissues with the most successful tissue-specific protein synthesizing mechanisms will form first and will be located at the primary sites of optimal conditions for protein synthesis. Subsequent differentiations would follow as conditions for protein synthesis became optimal in other areas and the preordained winners in the competition between other protein synthesizing mechanisms produced their populations of specific proteins. Such a mechanism would not only account for the appearance of tissues and organs at different times but would also account for their topographic placement. The formation of the head at the site of optimal conditions for protein synthesis in the flatworm would seem to provide some evidence in support of this speculation. The demonstration of gradients of $C^{14}O_2$ incorporation into proteins of the frog gastrula (Flickinger, 1954) and the induction of neural tissue from ventral ectoderm of the frog gastrula by acidic CO_2 shocks, which are thought to promote yolk utilization and subsequent protein synthesis (Flickinger, 1958a), also seem to fit into the framework of the hypothesis.

Summary

1. Intact planaria have been incubated with $C^{14}O_2$ or glycine C-14 and the protein fractions from various levels of the worms have been converted to CO_2 by combustion and collected as $BaCO_3$ and counted with a flow counter. An axial gradient of incorporation of $C^{14}O_2$ and glycine C^{14} into proteins has been revealed. The glycine gradient is modified by a slight increase in the tail region in comparison to the pharyngeal region. Experiments with different regions of regenerating worms have shown that although the sites of head and tail regeneration show an increased incorporation of glycine C-14 into proteins, the gradient pattern found in whole worms is maintained.

2. The utilization of antisera to anterior and posterior halves of flatworms in precipitin and Ouchterlony agar-plate diffusion experiments did not reveal any regional distribution of the main antigenic components of the flatworms.

3. Prior treatment of intact worms with M/20,000 colcemide (0.02 mg./cc.) (deacetylmethylcolchicine) for one day caused bipolar head formation in a large percentage of cut sections, thus confirming the work of Kanatani (1958). The presence of a head, in cut sections from colcemide treated worms, inhibited head formation at the posterior cut surface.

4. When cut sections of worms are embedded in agar, and the anterior ends placed in a chloramphenicol (1 mg./cc.) solution, the biological polarity is reversed in a significant percent of the cases as shown by head formation at the posterior ends of the sections. The presence of the head, even though immersed in chloramphenicol or colcemide, was found to inhibit the head formation at the posterior cut surface.

5. After treatment of intact worms with colcemide (0.02 mg./cc.) for one day, $C^{14}O_2$-colcemide incubation experiments revealed that this compound had obliterated, or altered, the normal axial gradient of $C^{14}O_2$ incorporation into proteins at the post-cephalic levels. Chloramphenicol (1 mg./cc.) treatment for 2 days greatly retarded the rate of $C^{14}O_2$ incorporation and altered the steepness of the normal gradient. Chloramphenicol inhibition suggests that $C^{14}O_2$ incorporation reflects synthesis of protein. It is assumed that chloramphenicol treatment of anterior ends of cut worms in the agar-slab experiments is inhibiting protein synthesis only at the exposed anterior ends of the worms since the unexposed posterior ends are subjected to only a very low concentration of inhibitor that diffuses through the agar.

6. The evidence suggests that reversal of a gradient of protein synthesis causes reversal of biological polarity in regenerating flatworms. Furthermore, it seems likely that this aspect of metabolic polarity, with its ancillary competition for nutrient, is a causal factor in the normal maintenance of polarity in regenerating planaria.

References

1. Björklund, B. 1952. Specific inhibition of precipitation as an aid in antigen analysis with gel diffusion method. *Proc. Soc. Exp. Biol. Med.*, **79**, 319-324.
2. Brønsted, H. V. 1955. Planarian regeneration. *Biol. Rev.*, **30**, 65-126.
3. Child, C. M. 1907. An analysis of form-regulation in Tubularia VI. The significance of certain modifications of regulation: polarity and form-regulation in general. *Archiv. für Entwick*, **24**, 317-349.
4. ————. 1941. Patterns and problems of development. Univ. of Chicago Press.
5. DuBois, F. 1949. Contribution à l'étude de la migration des cellules de régénération chez les Planaires dulcicoles. *Bull. biol.*, **83**, 213-283.
6. Flickinger, R. A. 1954. Utilization of $C^{14}O_2$ by developing amphibian embryos, with special reference to regional incorporation into individual embryos. *Exp. Cell. Res.*, **6**, 172-180.
7. ————. 1957. The relation between yolk utilization and differentiation in the frog embryo. *Amer. Nat.*, **91**, 373-380.
8. ————. 1958a. Induction of neural tissue in ventral explants from frog gastrulae by carbon dioxide shock. *Science*, **127**, 145-146.
9. ————. 1958b. Regional localization of neural and lens antigens in the frog embryo in relation to induction. *Biol. Bull.*, **115**, 201-208.

10. ————, AND BLOUNT, R. W. 1957. The relation of natural and imposed electrical potentials and respiratory gradients to morphogenesis. *J. Cell. and Comp. Physiol.*, **50**, 403-422.

11. GREGORY, F. G., & VEALE, J. A. 1957. A reassessment of the problem of apical dominance. *Symp. Soc. Exp. Biol.*, **11**, 1-20.

12. HYMAN, L. H., & BELLAMY, A. W. 1922. Studies on the correlation between metabolic gradients, electrical gradients and galvanotaxis. *Biol. Bull.*, **43**, 313-347.

13. HYMAN, L. H. 1932. The axial respiratory gradient: experimental and critical. *Physiol. Zool.*, **5**, 566-592.

14. KANATANI, H. 1958. Formation of bipolar heads induced by demecolcine in the planarian, Dugesia gonocephala. *Journ. Fac. of Science, U. of Tokyo.* Sec. 4, 8, Part 2, 253-270.

15. LENDER, T. 1956. L'inhibition de la régéneration du cerveau des Planaires, Polycelis nigra (Ehrb.) et Dugesia lugubris (O. Schm.) en présence de broyats de têtes ou de queues. *Bull. Soc. Zool.*, **81**, 192-199.

16. LOVTRUP, E. 1953. Studies on planarian respiration. *J. Exp. Zool.*, **124**, 427-434.

17. MARSH, G., & BEAMS, H. W. 1952. Electrical control of morphogenesis in regenerating Dugesia tigrina. *Jour. Cell. Comp. Physiol.*, **39**, 191-213.

18. MCWHINNIE, M., & GLEASON, M. M. 1957. Histological changes in regenerating pieces of Dugesia dorotocephala treated with colchicine. *Biol. Bull.*, **112**, 371-376.

19. MILLER, J. A. 1939. Experiments on polarity determination in Tubularia regenerates. *Anat. Rec.*, **75**, 4. Suppl.

20. ROUNDS, D. E., & FLICKINGER, R. A. 1958. Distribution of ribonucleoprotein during neural induction in the frog embryo. *J. Exp. Zool.*, **137**, 479-500.

Growth, 1959, **23**, 273-290.

EFFECT OF X-IRRADIATION ON ESTROGEN-INDUCED PROTEIN SYNTHESIS IN THE CHICK*

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INTRODUCTION

INVESTIGATORS from several laboratories have reported that, with the exception of serum albumin, serum protein synthesis occurring within liver cells of adult mammals is scarcely influenced by near-lethal doses of X-irradiation (1-4). It might be expected, however, that in the very young animal, in which liver cells are multiplying and/or differentiating, the effect of radiation upon protein synthesis by that organ would be more strongly expressed.

Earlier studies in this laboratory (5) have shown that irradiation (500-600r) is followed by decreases in *total* serum proteins in embryos and chicks despite the fact that cell division in the liver is unimpaired. However, the effects on serum proteins other than albumin have been variable. Lipoproteins have been observed to decrease in some age groups but to actually increase in others (5). In these cases the determining factors may have been synthesis of-and/or utilization of their lipid moieties.

In the present study an attempt has been made to approach radiation-inhibition of protein synthesis in the developing liver from a perspective made possible by a unique feature of the chick, namely, its ability to synthesize three new and different proteins of serum under the stimulus of estrogens (6-7). With this synthesis in young animals being initiated only by the application of such substances, it is possible to assay for the effect of irradiations with minimal interference arising from the presence of pre-existing protein. Further, if it can be assumed that systems for the synthesis of these moieties are

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absent or undeveloped in non-estrogenized chicks, a test of the radiosensitivity of developing enzyme systems may concomitantly be made.

Materials and Methods

Serum proteins elicited by estrogen treatment include serum X_1-phosphoprotein, serum X_2 lipoglycoprotein and a lighter lipoprotein (6-7). In the chick these are characterized by Svedberg constants S9.5, S20 and S_f 15-50, respectively. The phosphoprotein has been shown to carry most of the elevated calcium in the sera of these fowl. The lipoglycoprotein contains approximately 20 per cent lipid, mainly phospholipid, and the lipid moiety of the lighter lipoprotein (80% of the total molecule) consists mainly of sterol ester and phospholipid with varying amounts of triglyceride. Except in the case of the laying hen, these proteins are rarely present in the serum in significant concentration.

Experiment #1

Newly hatched chicks[1] were injected intraperitoneally with 2 to 6 mg. of estrogenic substances (Premarin, Ayerst Co., or Delestrogen, Squibb Co.) and were given 400, 600 or 750 r of X-irradiation on the same day.[2] The chicks were bled 1, 3, 4, or 5 days following such treatment. Estrogenized but non-irradiated, irradiated but non-estrogenized and untreated chicks, served as controls.

Pooled sera from each of the above experimental groups were assayed for (A) content of calcium (Table 1), and (B) content of light and dense lipoproteins, X_1-phosphoprotein and X_2-lipoglycoprotein as well as albumin and denser globulins. The amount of serum calcium in excess of the normal value (10 mg %) was taken as a reflection of the amount of X_1-phosphoprotein present in the serum (6-7). (In one case, analysis for protein (non-lipid) phosphorus was made as an additional indirect measure of the amounts of X_1 phosphoprotein present (Table 5).) Analyses of lipoproteins, X_1-phosphoprotein, X_2-lipoglycoprotein, albumin and dense globulins were carried out with the aid of the ultracentrifuge (Figs. 1-4 and Table 2). In some cases (Table 3) total lipids of sera were determined by the extraction procedures of Delsal (8) and constituent lipids by silicic acid chromatography (Table 4) (9).

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[1] The yolk sacs of some of these experimental and control chicks were removed surgically.

[2] Radiation factors were: 250 Kvp, 15 ma, 35 cm T.O.D., 0.21 mm Cu inherent, plus 0.5 mm parabolic Cu, plus 1.0 mm al filters, HVL = 1.9 Cu center of parabolic filter. Roentgens were measured in air

TABLE 1
TOTAL SERUM CALCIUMS

Exp. Number	Age Est.* Given	Age X-ray Given	Age Bled	Yolk Sac?	Food	No X-No Est.* Ca (mg o/o)	No X-Est.* Ca (mg o/o)	400 r-Est.* Ca (mg o/o)	600 r-No. Est.* Ca (mg o/o)	600 r-Est.* Ca (mg o/o)	750 r-Est.* Ca (mg o/o)
1	21 days**	21 days**	24 days**	Yes	Limited Mash		37.2			32.2	
1	21 days	21 days	24 days	Yes	No Food		16.9			15.8	
1	21 days	21 days	24 days	Yes	No Food		14.1			19.5	
1	21 days	21 days	24 days	No	Glucose		18.5			21.4	
1	21 days	21 days	24 days	No	Glucose		23.1			23.1	
1	21 days	21 days	24 days	No	Glucose		23.5			21.6	
1	21 days	21 days	24 days	Yes	No Food		11.7	11.3		9.6	9.8
1	21 and 23 days	21 days	24 days	Yes	Limited Mash	11.2	26.5	32.1		31.2	27.8
1	21 and 23 days	21 days	26 days	Yes	Limited Mash	11.2	24.8	30.4		27.8	33.4
1	21 and 23 days	21 days	26 days	Yes	Limited Mash	11.2	34.1	25.1		24.2	28.5
2	21 days	17 days	24 days	Yes	Limited Mash		28.0			26.7	
2	21 days	18 days	24 days	Yes	No Food		15.1			20.2	
2	21 days	18 days	24 days	Yes	No Food	14.0	16.3		9.8	17.6	
2	21 days	19 days	24 days	Yes	Limited Mash	15.2	26.3		14.0	24.6	
2	21 days	20 days	24 days	Yes	No Food		17.1		8.6	19.7	
							Mean 22.2			Mean 22.3	
Control	6 mo.***			No							
	(Roosters)	6 Mo.	6 mo. + 3 days		Mash		35.0				
	(Roosters)	6 Mo.	6 mo. + 3 days		Mash		47.2				

* Estrogen.

** Refers to number of days elapsed since eggs placed in incubator. Eggs hatch at 21 days—hence "21 days" indicates newly hatched chicks and "24 days" indicates chicks 3 days post-hatching.

*** Approximate age.

TABLE 2

TOTAL LIGHT LIPOPROTEIN (S_f 0-100)

Exp. Number	Age Est.* Given	Age X-ray Given	Age Bled	Yolk Sac?	Food	No X-No Est.* Lipo. (Rel. Units)**	No X-Est.* Lipo. (Rel. Units)**	400 r-Est.* Lipo. (Rel. Units)**	600 r-No Est.* Lipo. (Rel. Units)**	600 r-Est.* Lipo. (Rel. Units)**	750 r-Est.* Lipo. (Rel. Units)**
1	21 days***	21 days***	24 days***	No	Glucose	0	24			22	
1	21 days	21 days	24 days	No	Glucose		16			10	
1	21 days	21 days	24 days	No	Glucose		7			17	
1	21 days	26 days	27 days	Yes	Limited Mash		18			15	
1	22 days	27 days	29 days	Yes	No Food		5			5	4
	22 and 24 days	22 days	25 days	Yes	Limited Mash		15	15		11	11
	22 days	22 days	25 days	Yes	No Food		6	6		5	6
1	21 and 23 days	25 days	26 days	Yes	Limited Mash		29	33		37	35
1	21 and 23 days	21 days	24 days	Yes	Limited Mash		28	37		38	37
1	21 and 23 days	21 days	26 days	Yes	Limited Mash	3	25	22	2	24	25
2	21 days	12 days	24 days	Yes	Limited Mash	1	22			19	
2	21 days	17 days	24 days	Yes	Limited Mash		16			19	
2	21 days	18 days	24 days	Yes	No Food	0	3			3	
2	21 days	19 days	24 days	Yes	Limited Mash	2	19		2	20	
2	21 days	20 days	24 days	Yes	No Food	2	15			14	
							Mean 16.5			Mean 17.2	

* Estrogen (2 mg per dose).

** Relative Units. Actually, the number of square millimeters of area occupied by the lipoprotein deflection.

*** Refers to number of days elapsed since eggs placed in incubator. Eggs hatch at 21 days—hence "21 days" indicates newly hatched chicks and "24 days" indicates chicks 3 days post-hatching.

TABLE 3
TOTAL SERUM LIPIDS

Exp. Number	Age Est.* Given	Age X-ray Given	Age Bled	Yolk Sac	Food	No X-No Est.* Lipid (Mg/ml)	No X-Est.* Lipid (Mg/ml)	400 r-Est.* Lipid (Mg/ml)	600 r No. Est.* Lipid (Mg/ml)	600 r-Est.* Lipid (Mg/ml)
1	22 days**	22 days**	25 days**	Yes	Limited Mash	15.6	25.4		19.2	35.9
1	22 days	22 days	25 days	Yes	Limited Mash	15.4	30.6		17.8	35.4
1	21 days	21 days	24 days	Yes	No Food		15.6	16.4	14.4	14.6
2	21 days	17 days	24 days	Yes	Limited Mash		29.4			32.4
2	21 days	19 days	24 days	Yes	Limited Mash	20.0	32.0		22.6	34.0
2	21 days	20 days	24 days	Yes	No Food		32.6		22.6	35.2

* Estrogen.

** Refers to number of days elapsed since eggs placed in incubator. Eggs hatch at 21 days—hence "21 days" indicates newly hatched chicks and "24 days" indicates chicks 3 days post-hatching.

TABLE 4
CONSTITUENT SERUM LIPIDS OF CONTROL, ESTROGENIZED AND ESTROGENIZED-IRRADIATED CHICKS

	Sterol Ester %*	Triglyceride %*	Sterol %*	Phospholipid %*
No X-No Est.**	50.0 (48.3–51.7)[2]	2.5 (2.5)[2]	7.9 (7.6–8.2)[2]	39.6 (38.2–41.0)[2]
600 r*** No Est.**	46.1 (44.0–50.0)[2]	2.4 (2.0–2.9)[2]	3.9 (2.0–5.9)[2]	47.6 (41.2–54.0)[2]
Estrogen (4 mg)	20.8 (20.4–21.3)[2]	40.0 (39.0–41.0)[2]	5.9 (5.3–6.6)[2]	33.1 (31.1–35.2)[2]
Estrogen-600 r	20.3 (19.8–20.9)[2]	39.9 (39.2–40.6)[2]	6.4 (6.1–6.6)[2]	33.4 (33.0–33.7)[2]
Estrogen-750 r	19.6 (19.1–20.2)[2]	39.8 (39.5–40.2)[2]	8.6 (7.2–10.2)[2]	31.8 (30.5–33.0)[2]

* Percent of Total Lipid.
** Estrogen.
*** Chicks killed 3 days post-irradiation.
Figures in parentheses represent range. Superscript indicates number of analyses.

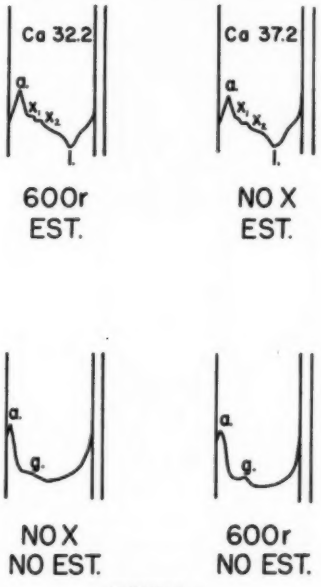

FIGURE 1

Typical ultracentrifuge patterns of chick samples with and without radiation and estrogen taken three days post-injection with 2 mg. estrogen. X-irradiation was given on the day of estrogen treatment. Sedimentation of albumin, X_1 and globulin is toward the right. Lipoproteins are floating towards the left in each pattern. Sera were diluted with one part 17.6% NaCl. Pictures were taken 32 minutes after reaching speed (50740 rpm). Legend: a = albumin, g = globulin, l. = light lipoprotein, $X_1 = X_1$ — phosphoprotein, $X_2 = X_2$ — lipoglycoprotein.

Note that the albumin erroneously appears to be slightly increased following irradiation in both estrogenized and non-estrogenized birds. This is due to the presence of additional dense lipoprotein in irradiated chicks. Calcium values are in milligrams per cent.

EXPERIMENT #2

Since factors associated with the stages of development of the liver could possibly determine its response to irradiation (Fig. 5 shows that there is a doubling in the size of the liver between 18 days of incubation and the time of hatching, whereas there is scarcely any increase in size during the first 4 days post-hatching) groups of embryonated eggs of 12, 17, 18, 19 and 20 days of incubation were also irradiated

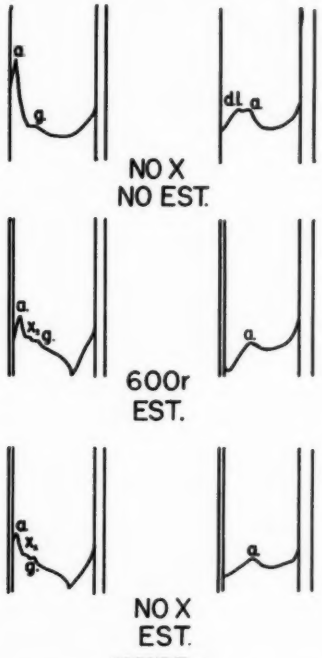

FIGURE 2

Typical ultracentrifuge patterns of chick serum with and without radiation and estrogen, but taken 7 days post-injection with 4 mg estrogen (Ayerst). X-irradiation was given 24 hours before killing. Sera were diluted with 1 part 17.6 per cent NaCl. Pictures were taken 16 and 176 minutes after reaching speed (50740 rpm). Legend: a = albumin, g = globulin, d.l. = dense lipoprotein, $X_2 = X_2$ — lipoglycoprotein.

Note that the albumin and the dense lipoprotein are both decreased as a result of estrogen treatment but that irradiation has had no detectable effect in this short interval on any component, including albumin.

(Tables 1, 2, and 3). Estrogen treatment was initiated immediately after hatching and the chicks were bled three days following injection of estrogen.

EXPERIMENT #3

In order to ascertain the effect of irradiation on rates of serum protein *turnover,* irradiated and control chicks were given intraperitoneal injections of S^{35}-labeled methionine or cysteine and were bled

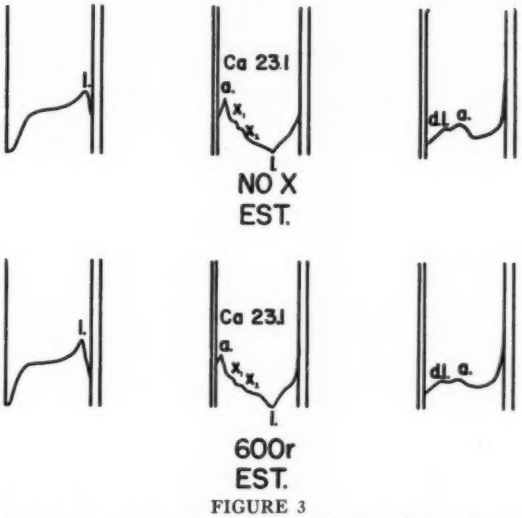

FIGURE 3

Change in ultracentrifuge patterns with centrifuging time for samples of chick serum taken 3 days post-injection with 2 mg. estrogen. X-irradiation given on the day of estrogen treatment. These sera were diluted with one part 17.6 per cent NaCl solution. Pictures were taken 0, 32 and 176 minutes after reaching speed (50740 rpm). Patterns in the frames to the far left are upside down so that the peak remains within the picture. Legend: a = albumin, d.l. = dense lipoprotein, l. = light lipoprotein, X_1 = X_1 — phosphoprotein, $X_2 = X_2$ — lipoglycoprotein.

Note (in the frames to the far right) that X-irradiation resulted in an increase of dense lipoprotein and a decrease in serum albumin. Note also (in the frames to the far left) that concentrations of S_f 10-100 lipoproteins were very similar whether the chicks were irradiated or not.

at various intervals after injection. Counts of radioactivity were made on the whole sera (Figs. 6 and 7), and also on the lighter lipoproteins (Fig. 8) subsequent to their resolution by paper electrophoresis. Injections of various labeled or otherwise identifiable proteins were also compared in irradiated and control chicks with respect to rates of clearance from the serum (Table 6).

Results

As illustrated by ultracentrifuge patterns in Figs. 1-4 and by data presented in Table 2, estrogen treatment was always followed by

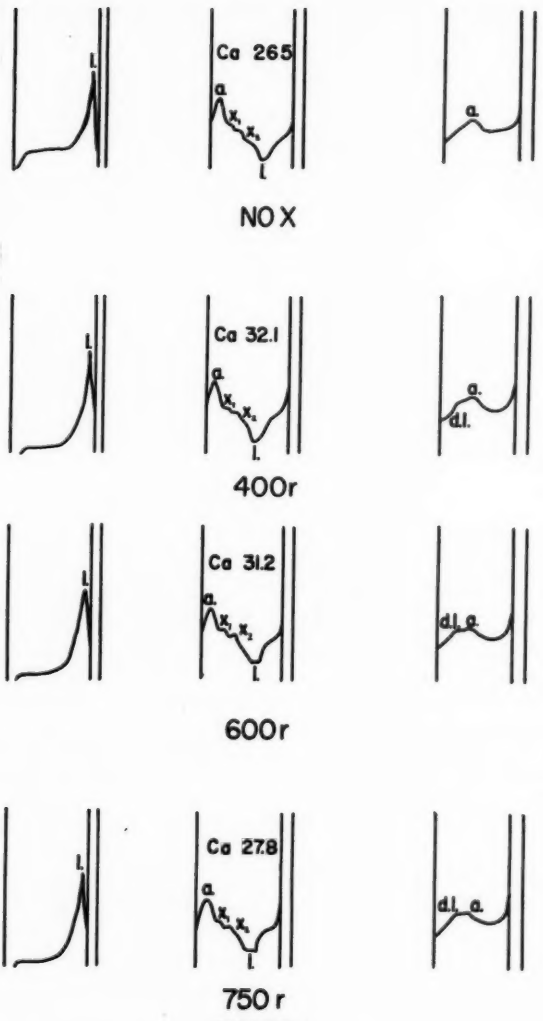

FIGURE 4

Effect of radiation dose on ultracentrifuge patterns of chick serum taken three days post-injection with 2 mg. estrogen and one day post-injection with 2 additional mg. estrogen. Radiation was administered on the day of the first estrogen injection. Pictures were taken, 0, 48 and 176 minutes after reaching speed (50740). Sera were diluted with one part 17.6 per cent NaCl solution. Patterns in the frames to the far left were turned upside down. Legend: a = albumin, d.l. = dense lipoprotein, l. = light lipoprotein, $X_1 = X_1$ — phosphoprotein, $X_2 = X_2$ — lipoglycoprotein.

Note (in center frames and frames to the far right) that albumin was decreased slightly by irradiation below the decrease brought about by estrogen-injection and that dense lipoprotein was increased following irradiation.

TABLE 5
TOTAL SERUM NON-LIPID PHOSPHORUS

Age Est.* Given	Age X-ray Given	Age Bled	Yolk Sac	Food	No X-No Est.* P	No. X-Est.* P	400 r-Est.* P	600 r-No Est.* P	600 r-Est.* P	750 r-Est.* P
21 and 23 days**	21 days**	24 days**	Yes	Limited Mash	0.57 o/o	3.2 o/o	3.6 o/o		4.2 o/o	3.8 o/o

* Estrogen.
** Refers to number of days elapsed since eggs placed in incubator. Eggs hatch at 21 days—hence "21 days" indicates newly hatched chicks and "24 days" indicates chicks 3 days post-hatching.

TABLE 6
INTRACIRCULATORY INJECTION EXPERIMENTS

Days after 600 r	Material Injected	Hrs. after Injection	Method of Analysis	No X-ray	X-ray
3	S^{35} Beta	2½	Activity Count	101	110
3	S^{35} Alpha	2½	Activity Count	80	78
3	W. S. (Tung Oil)	4	Sprectrophotometer	1.0	1.0
3	W. S. (Tung Oil)	4	Sprectrophotometer	.23	.23
3	Beta (Est. Rooster)	4	Ultracentrifuge	66%	66%
3	Egg Yolk Fluid	8	Lipid Extraction	15.0 mg/ml	11.4 mg/ml
3	S^{35} Beta	9	Activity Count	.10 c/s	.074 c/s
3	W. S. (Est. Rooster)	9	Lipid Extraction	12.5 mg/ml	13.2 mg/ml
3	Egg Yolk Fluid	9	Lipid Extraction	20.6 mg/ml	22.2 mg/ml
3	Egg Yolk Fluid	10	Lipid Extraction	17.4 mg/ml	15.0 mg/ml

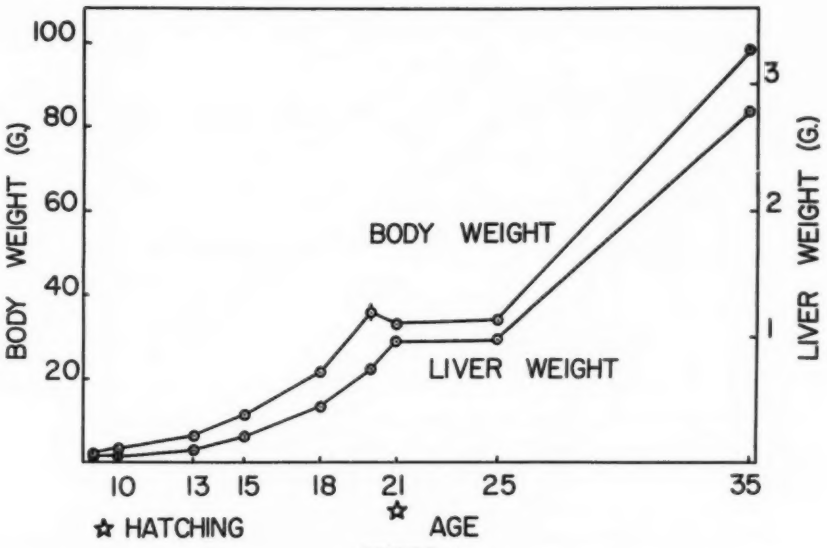

FIGURE 5

Total wet weight of the embryo and its liver during development. Vertical lines through the points represent twice the standard deviation for the group.

Note that the standard deviations in most cases are so small that they fall within the point-circle.

increases in serum concentration of lighter lipoproteins and serum X_2-lipoglycoproteins (within 24 hours). In each case, where X_2 was elevated, serum albumin and the normally occurring dense lipoproteins of serum (-S 0-10) were decreased. It was not convenient to quantitatively measure the serum X_1-phosphoproteins in these patterns due to their relatively low concentrations. That levels of these moieties were nonetheless increased by estrogen treatment was indicated by significant rises in serum calcium (Table 1) and non-lipid serum protein phosphorus (Table 5).

There was, of course, much more lipid in the sera of estrogenized chicks (Table 3) as compared to control chicks. The major difference in proportions of constituent serum lipids of estrogenized chicks as compared to non-estrogenized chicks was a large increase in serum triglycerides (Table 4).

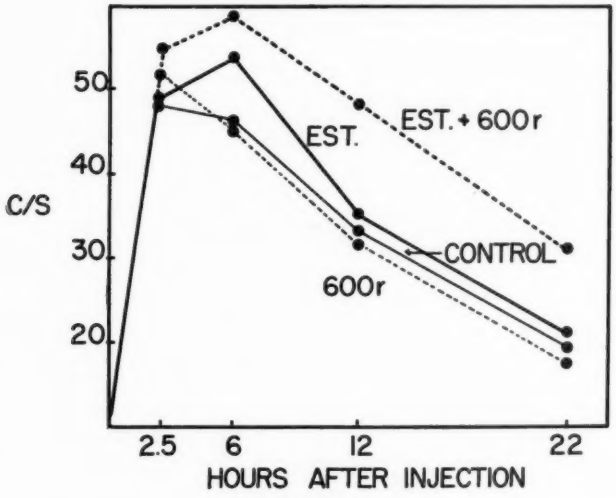

FIGURE 6

Radioactivity (C/S = counts per second) of pooled whole sera of chicks (4 chicks per point) at various times following the intraperitoneal injection of S^{35}-labeled methionine.

In this non-typical experiment there was an increase in synthesis of S_f 15-50 lipoprotein in the estrogenized-irradiated chicks. This largely accounts for the unusually increased uptake of S^{35} in hte sera of estrogenized-irradiated chicks.

As indicated in Tables 2 and 4, actual synthesis of triglyceride probably takes place under stimulus of estrogen, since this lipid appeared in relatively large amounts in the serum, even with removal of the yolk sac, which is the primary store of triglyceride in the newly hatched chick.

Under none of the experimental conditions, nor in any of the stages studied, was X-irradiation (400-750 r) followed by detectable changes in serum concentrations of estrogen-induced lighter lipoproteins (Table 2), X_2-lipoglycoproteins (Figs. 1-4) or X_1-phosphoproteins (Tables 1 and 5). Normal dense lipoproteins were, however, usually increased following irradiation as indicated by statistically significant (P. 95) increases in total serum lipids (Table 3), and by comparison of the ultracentrifuge patterns (Figs. 3 and 4). A difference in proportions of constituent serum lipids must, therefore, be present in

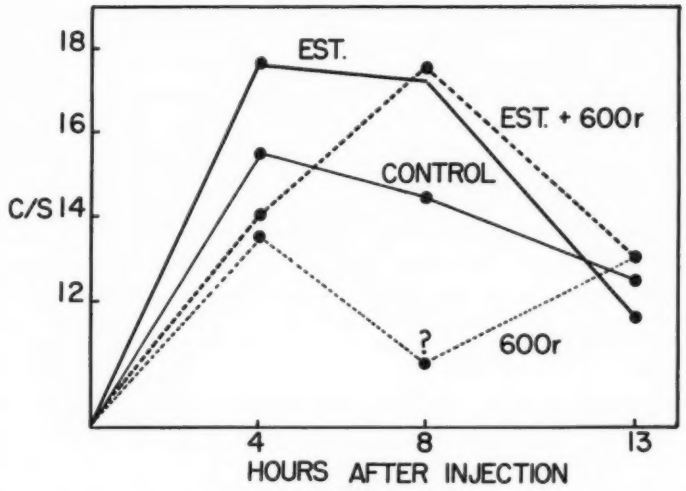

FIGURE 7

Radioactivity (C/S = counts per second) of pooled whole sera of chicks (4 chicks per point) at various intervals following the injection of S^{35}-labeled cysteine.

irradiated estrogenized and non-irradiated estrogenized chicks but this is so slight that it escapes detection by the methods used (Table 4). Slight additional decreases following irradiation were detected with respect to concentrations of the serum albumin that had already been lowered because of estrogen (Figs. 2, 3 and 4). Total turnover, either of native serum proteins (Figs. 6, 7 and 8) or of various types of proteins injected into the circulation via the heart, seemed relatively unaffected by the radiation doses administered (Table 6), but turnover of certain proteins was, of course, increased by the injection of estrogen alone.

Discussion

These results clearly indicate that the serum concentrations of those proteins whose synthesis is initiated and stimulated by the presence of estrogens, are not detectably altered in young animals by near-lethal nor even by lethal doses of irradiation when measured 1 to 12 days post-irradiation. It is possible that some depressive responses might have been obtained had X-irradiation been followed immedi-

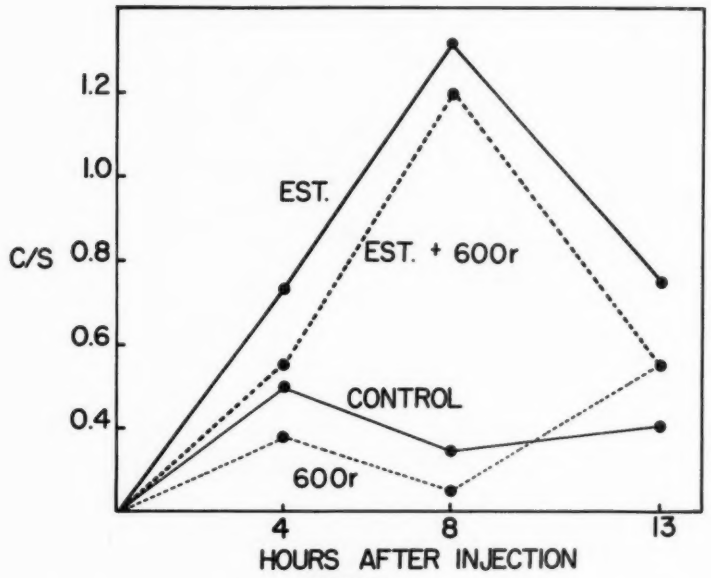

FIGURE 8

Radioactivity (C/S = counts per second) of pooled beta lipoproteins (S_f 15-50) (4 chicks per point) at various intervals following the injection of S^{35}-labeled cysteine. The original serum for this analysis was the same as that taken for the experiment depicted in Figure 7.

ately by estrogen stimulation in those embryos that were irradiated at 12 to 20 days of incubation (with the embryos being sacrificed at the usual 3-5 day interval following estrogen stimulation). This, however, was impractical and it should be pointed out that any recovery processes that might have occurred with time (i.e., before estrogen was administered) could not have involved cell division as this was unaffected by the dosage employed.

Other results have indicated that protein synthesis *per se* is often very resistant to irradiation, even in the developing organism. A striking example is that given by Allen, *et al.* (10), who found that pituitary cells of tadpoles continued to synthesize thyrotrophic hormone (a protein substance) as long as the cells remained intact following a dosage of 200,000 r.

It is also possible that the development of the enzyme system (including ribonucleoprotein) involved in the production of these specific proteins is relatively unaffected by these doses of irradiation. Such a conclusion must, however, be based on the assumption that the enzyme systems were not developed before the estrogen was given. In this connection, Straub, *et al.* (11), have found that liver normally contains a large amount of inactive, masked tryptophane peroxidase. Under conditions such as paper electrophoresis of liver extract, tryptophane peroxidase activity increases 30-40 fold as a result of enzyme de-masking. Although the enzymes concerned with the synthesis of the serum proteins in question might also be already present in masked form, it is pointed out that the embryonic liver at 12 days of incubation is only one-fifteenth of its weight at hatching and that even the 17 day liver possesses only one-third its complement of cells at the time of hatching. In terms of cytoplasmic ribonucleoprotein, the increase during development must be several times greater since the *total* microsomal fraction per cell is increased by approximately one-third between the 12th and 24th day. It is not decreased following irradiation (12).

Concentrations of dense globulins (including gamma globulin) are so low in the chicken embryo and young chick that changes following irradiation are difficult to follow. The literature offers abundant evidence that the gamma globulin of the serum is often depressed following irradiation but in the case of this serum protein the effect would seem to be based on the fact that the continuity of the somewhat special source of these proteins, namely, the plasma cells, is destroyed. Previous studies in this laboratory (13) have shown that serum concentrations of mucoproteins are unaffected by administration of 500-600 r.

Higher concentrations of dense lipoproteins were always seen after irradiation of either estrogenized or non-estrogenized animals. These increases do not seem to occur at the expense of X_1, X_2 or of the lighter lipoproteins.[3]

[3] Because of an apparent correlation between the occurrence of X_2 lipoglycoprotein and decreased serum concentrations of dense lipoprotein after administration of estrogen, it could be suspected that X_2-lipoglycoprotein was derived from the superficially similar dense lipoprotein. This seems hardly to be the case, however, when the haphazardness of the correlation is exposed, as by time sequence studies (14). There is likewise no consistent correlation between the disappearance of dense lipoproteins and the appearance

Summary

Under none of the experimental conditions nor in any of the stages studied was whole body X-irradiation (400-750 r) followed by significant changes in serum concentrations of estrogen-induced lighter lipoproteins, X_2-lipoglycoproteins or X_1-phosphoproteins. Small but statistically significant increases in total serum lipids and dense lipoproteins were observed, as were decreases in serum albumin. No indications of gross changes in turnover of serum proteins (labeled with methionine- or cysteine-S^{35}) were detected following whole body irradiation.

References

1. Goldwater, W. H., & Enteman, C. (1957). Nature of serum protein changes in X-irradiated dog. *Am. J. Physiol.*, **188**, 409-414.
2. Meynill, S., & de Mende, S. (1955). Electrophoretic study of serum proteins of irradiated animals. *Bull. Soc. Chim. Biol.*, **37**, 127-131.
3. Fischer, M. A., Magee, M. Z., & Coulter, E. P. (1955). Studies on the serum proteins of the X-irradiated rat. *Arch. Biochem. Biophys.*, **56**, 66-74.
4. Leone, C. A., Hartnett, A. R., Crist, R., & McBeth, CaroLee. (1959). Paper electrophoretic analyses of sera from irradiated rhesus monkeys. *Radiation Research*, **10**, 357-369.
5. Schjeide, O. A., Ragan, Nancy, & Simons, Sue. (1958). The effect of X-irradiation on plasma lipoprotein patterns in the embryo. *Radiation Research*, **9**, 327-340.
6. ———, & Urist, M. R. (1956). Proteins and calcium in serums of estrogen-treated roosters. *Science*, **124**, 1242-1244.
7. Urist, M. R., Schjeide, O. A., & McLean, F. C. (1958). The partition and binding of calcium in the serum of the laying hen and of the estrogenized rooster. *Endocrinology*, **63**, 570-585.
8. Delsal, J. L. (1944). New procedure for extraction of serum lipids with methylal. *Bull. Soc. Chim. Biol.*, **26**, 99-105.
9. Fillerup, D. L., & Mead, J. F. (1953). Chromatographic separation of plasma lipids. *Proc. Soc. Exptl. Biol. Med.*, **83**, 574-577.
10. Allen, B. M., Schjeide, O. A., Piccirillo, R. T., & Kowitz, G. (1953). The capacity of the anterior pituitary gland to function after heavy irradiation. *J. Exptl. Zoo.*, **124**, 131-145.
11. Straub, F. B. (1956). Minutes of the Internat. Conference on Proteins (Libice, Czechoslovakia), p. 212.
12. Schjeide, O. A., & Ragan, Nancy. Effects of X-irradiation on cellular inclusions in chick embryo livers. Under preparation.
13. ———, & Simons, Sue. (1958). Lack of glycoprotein response in irradiated chicken embryos. *Growth*, **22**, 265-268.
14. ———, & Simons, Sue. Effects of estrogen on serum glycoproteins and seromucoids. Under preparation.

of lighter lipoproteins, nor does any precise relationship seem to exist between X_2 and the lighter lipoproteins, although both of these are *usually* elevated at the same time following estrogen treatment.

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